USING THE INFLUENZA POLYMERASE 5'-ENDONUCLEASE ACTIVITY TO DEVELOP NOVEL siRNA THERAPEUTICS

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ABSTRACT

Influenza is a global public health burden, producing seasonal epidemics with 3-5 million severe cases and 250,000 to 500,000 deaths each year. In addition to seasonal illness, influenza has also been responsible for global pandemics, the most notorious being the 1918 "Spanish Influenza." While vaccination is the most useful tool for seasonal or pandemic influenza control, such interventions would not help individuals who are already infected. Antiviral drugs such as neuraminidase inhibitors and adamantanes can be effective if taken soon after symptoms appear, but antiviral resistance is developing.

An alternative to antiviral drugs is the promising field of therapeutics based on RNA interference (RNAi). RNAi based treatments utilize chemically synthesized short interfering (si)RNAs between 21-24 nt in length to silence genes of interest. A number of research groups have investigated RNAi based antivirals, but the main limitations to their clinical use are risks of off-target effects and difficulty with appropriate dose delivery. A potential solution to both problems would be to use a pro-siRNA which is inert in uninfected cells but activated upon infection, thus limiting off-target effects to infected cells while sparing healthy cells. This approach would also provide dosing flexibility, as the active dose would be modulated by viral activity. In the case of influenza, a pro-siRNA consists of a standard siRNA duplex with a 10-13 nucleotide 7-methyl-guanosine (M_7G) capped sequence on the 5' end of the passenger or positive sense strand. This extension should render the pro-siRNA biologically inert in uninfected cells but be cleaved off in infected cells via PA mediated cap snatching activity, leaving behind a functional siRNA duplex.

To test the feasibility of this approach, we designed variants of pro-siRNAs targeting eGFP, which can be expressed in a variety of cell types using transient or stable transfection. Cells were transfected with eGFP producing plasmids and/or siRNAs, infected with influenza 24-26

hours post transfection and fixed and analyzed 24 hours post infection. Through use of flow cytometry, we found that several pro-siRNA designs produced modest reductions in eGFP expression in infected cells, suggesting that the concept is feasible. However, further optimization will be required for capped pro-siRNAs to be a practical antiviral approach against seasonal and pandemic influenza.

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CHAPTER 1
General Introduction

Influenza Biology

Influenza viruses are enveloped, negative sense single strand RNA viruses in the family of *Orthomyxoviridae*, with genomes consisting of between 6 and 8 segments (1). The influenza genus contains several subtypes; aptly named influenza A, B and C (1). The influenza life cycle begins with attachment to the host cell surface via an α2,3 or α2,6 sialic acid residue, which the virus uses as its receptor. The virus is taken up via endocytosis where a decrease in endosomal pH causes a conformational change in its hemagglutinin (HA) protein, resulting in fusion and endosomal escape (1). At the same time, protons from the increasing acidity of the endosome pass through the M2 ion channel (in influenza A viruses) to the capsid interior, resulting in the pH mediated dissolution of the capsid matrix and release of the viral ribonucleoproteins (RNPs) (1, 2).The RNPs traffic to the nucleus where viral replication takes place. Hemagglutinin, neuraminidase and M2 proteins are produced and then incorporated into the plasma membrane of the host cell (2). Copies of each gene segment are then incorporated into RNPs which leave the nucleus while complexed with M1 protein (2). Virus particles form at the plasma membrane where they then bud through, acquiring an envelope studded with hemagglutinin and neuraminidase. Neuraminidase ensures efficient release of virus particles by cleaving sialic acid residues at the budding site to which the HA protein otherwise binds (2). The virions are now able to infect other cells.

It should be emphasized that influenza is unique among single strand RNA viruses in that it replicates in the nucleus as opposed to the cytosol (1). This is primarily due to its dependence on host RNA splicing machinery (3), but also its replication strategy of utilizing caps from host pre-mRNAs, which are confined in the nucleus until 3' end cleavage and polyadenylation take

place (2). Cellular mRNAs carry an N-7-methylguanosine (M_7G) cap at their 5' end, which protects against degradation by 5' exonucleases and aids in mRNA translation (4). As the pre-mRNA is produced, the 5' end is capped enzymatically (4). Following polyadenylation, the cap is bound by the nuclear cap binding complex, which facilitates mRNA export to the cytosol (4). The cap is then bound by eIF4E to initiate ribosome formation at the 5' end of the mRNA (5). In order for viruses to replicate in their host cells, they must develop ways to get around this mechanism. An example is the caliciviruses, which use the cap protein Vpg on the 5' ends of its RNAs so that they can bind eIF4E for translation (6). While picornaviruses also carry Vpg, they encode an Internal Ribosome Entry Site (IRES) in their RNA that allows for cap-independent translation by host ribosomes (1). Influenza uses a different strategy to get around this problem, utilizing 5' caps from the host to enable transcription and translation of its mRNAs. While other cap snatching viruses steal caps from host mRNAs in the cytosol (7), influenza utilizes host pre-mRNAs, which confines its replication and transcription activities to the nucleus.

The process of influenza RNA replication and transcription is carried out by the polymerase complex, consisting of PB1, PB2 and PA (8). The polymerase activity is located in the PB1 subunit, while cap snatching is carried out by the combination of PB2, which binds the cap, and PA, which serves as the endonuclease (9). Upon import into the nucleus, each gene segment is encased in an RNP that contains a copy of the polymerase complex (8). These must be included in every infectious virus particle because the negative sense RNA gene segments cannot be readily translated by host ribosomes and therefore must be transcribed to produce viral proteins (1). PB2 binds the 5' cap of a cellular pre-mRNA, while the PA endonuclease cleaves 10-13 nucleotides downstream (9), resulting in a capped oligonucleotide that remains bound to PB2. The capped oligonucleotide is then used by PB1 as a primer for transcription of a viral RNA.

Before releasing the transcript, the polymerase encounters a U rich sequence causing it to 'stutter', which produces a 3' poly(A) tail (10). The resulting chimeric, capped and polyadenylated viral mRNA can now be exported from the nucleus and translated by host ribosomes (8).

Influenza replication is further mediated by the NS1 protein, which is the most abundantly produced protein in infected cells (11). NS1 has multiple functions, including the supression of host gene expression (3). It does this by binding the cellular Cleavage and Polyadenylation Specificity Factor (CPSF) and Poly(A)-Binding protein II (PABII) (11, 12). This effectively inhibits the 3' processing and polyadenylation of host pre-mRNAs, preventing their export from the nucleus and providing an ample supply of caps to steal (2). Because influenza mRNAs are polyadenylated by the viral polymerase, they can still be exported from the nucleus and translated. Such a mechanism reduces host competition for cellular resources, vastly favoring virion production.

There are two major aspects of influenza replication that present epidemiological challenges. The first of these is a phenomenon known as antigenic drift. Like in most RNA viruses, the influenza RNA dependent RNA polymerase lacks a proofreading mechanism, resulting in a higher rate of mutation during transcription and viral replication (1). While most viral proteins are not as tolerant to these mutations and are therefore conserved (13), the HA and NA proteins mutate frequently (14).The gradual change in HA epitopes over time allows for the evasion of host adaptive immune responses, and is one of the main reasons why individuals can be infected year after year, and why new influenza vaccines are required each year.

While antigenic drift contributes to the yearly occurrence of seasonal flu, a phenomenon called antigenic shift contributes to the appearance of pandemics. As new virions are assembled, each

of the required gene segments is incorporated into the capsid. When multiple viruses infect the same cell, gene segments from either virus can be packaged into progeny virions, leading to a novel virus with different surface antigens (15). A pandemic can occur when an avian virus reasserts with a human virus to give a virus with the ability to replicate in humans but carrying an HA protein to which the human population has no residual immunity (15).

Public Health Burden of Seasonal Influenza

Influenza is a global public health burden, with seasonal epidemics producing 3 to 5 million severe cases and between 250,000 and 500,000 deaths annually worldwide (16). Those at highest risk for developing complications or requiring hospitalization for influenza illness are young children, individuals who are 65 years of age or older, women who are pregnant, and individuals with chronic health conditions such as diabetes, lung diseases or heart problems (16, 17). Influenza is transmitted by the emission of respiratory droplets from sneezing or coughing, which are transferred to others directly through inhalation, or indirectly through contact with a contaminated surface followed by touching of the nose or mouth (17). Most influenza infections present as respiratory illness, affecting the nose, throat, trachea, bronchi and occasionally deeper into the lung (16). Symptoms of a typical influenza infection include fever, chills, malaise, head and muscle aches and sore throat (16, 17). While these are often seen in other illnesses, their association with widespread annual influenza epidemics earns them the categorization of 'flu-like symptoms'. The most common complication is viral or bacterial pneumonia, with renal failure and neuromuscular symptoms having also been reported though rare (18).

Pandemic influenza

In addition to seasonal illness, influenza also has been known to cause global pandemics. The first known influenza pandemic occurred in 1580 and historical records also show that four pandemics occurred during the 19th century (17). The worst pandemic in contemporary history is the 1918 H1N1 "Spanish Influenza", which affected between 20 to 40% of the entire world population and killed an estimated 50 Million people (19). Subsequent pandemics in the 20th century include the 1952 H2N2 "Asian flu" and the 1968 H3N2 "Hong Kong" Influenza (19). The most recent pandemic was the 2009 H1N1 "Swine Flu", which the CDC has estimated to have caused around 284,000 deaths worldwide (20).

While the three previous influenza pandemics have been relatively light (19), there is still the potential for emerging influenza viruses to cause a devastating global pandemic on the same level or worse than seen in 1918. Two emerging avian influenza viruses, H5N1 and H7N9 have been closely monitored since their detection. The highly pathogenic H5N1 virus was first seen in chickens in Guangdong, China in 1996, followed by a much larger outbreak in Hong Kong in 1997, in which the disease spread to humans (21). As of 2012, there have been 610 cases of H5N1 influenza with 309 deaths reported since 2003. (21). The H7N9 virus emerged in 2013 in China, causing 126 cases with 24 deaths. Neither of these viruses has shown sustained human to human transmission (21, 22), but reassortment with a human influenza virus could increase the ability of the virus to transmit between humans, resulting in a global pandemic with significantly higher morbidity and mortality than was observed in 1918.

Current options for Influenza pandemic/seasonal control

During seasonal epidemics and the occasional pandemic, common means of influenza control include epidemiologic countermeasures such as frequent hand washing and social distancing.

The most effective means of control we currently have however is vaccination. Though there are multiple formulations available, influenza vaccines currently fall into two categories, which are inactivated (TIV) and live attenuated influenza vaccines (LAIV) (17). The inactivated vaccines are generally produced via formalin inactivation of virus particles, and are typically delivered by intramuscular or intradermal injection (17). The advantage of the inactivated vaccines is that they can be administered to children as young as six months of age, as well as the pregnant women and the elderly, all of which are major risk groups during flu season (17). The major disadvantage of the inactivated vaccines is the risk of Guillain-Barré Syndrome, a neurological autoimmune disease that has been previously associated with influenza vaccination and infection (23). While this is an extremely rare occurrence on the individual level, it is a concern when vaccinating millions of people as part of a pandemic response.

In contrast to the inactivated vaccines, the LAIV is administered intranasally and consists of viruses that have been attenuated via reassortment with a vaccine strain that has been cold adapted through repeated passage in tissue culture at 32 degrees Celsius (24). The resulting viruses replicate efficiently in the nasal passages and upper respiratory tract, but replicate less efficiently in the warmer lower respiratory tract (17, 24). While not showing increased efficacy in adults, LAIV have shown increased efficacy in children as compared to the inactivated vaccines (17). The main disadvantage of live attenuated influenza vaccines are that they cannot be given to pregnant women, children younger than 2, or adults older than 49 years of age (17), which excludes those at highest risk of complications during seasonal flu epidemics.

While useful for prevention and control of seasonal and pandemic influenza, both types of influenza vaccine are limited by their means of production. The vast majority of influenza vaccines currently licensed in the United States are produced by growing the vaccine viruses in

embryonated chicken eggs, a method that has been in use since the 1940s (25). Major issues with this production strategy include the infrastructure to produce large quantities of pathogen-free eggs from biosecure chicken flocks (26), and the low yield of vaccine per egg (27). The biggest drawback to current vaccine production is the lengthy timetable. Production of the first vaccine virus from isolates of interest can take several weeks while optimization of conditions for growth in eggs can take several weeks more (28). Producing sufficient doses of vaccine for flu season generally takes at least six months from virus isolation to final packaging of vaccines for distribution (28). As a result of such a timescale, vaccine developers must make predictions about what viruses will be prominent next season, based on isolates from previous seasons. An incorrect guess can result in a 'mismatch year', in which the vaccine does not adequately protect from the dominant strains circulating that particular season. A notorious example of vaccine mismatch is the 2003-04 season, when the A/Panama/2007/99 virus was selected as the H3N2 component of the 2003 vaccine, but the majority of current H3N2 isolates resembled the antigenically distinct A/Fujian/411/2002 virus (29). The mismatch contributed to the increased morbidity and mortality observed in the 2003-04 flu season, which was more severe than the previous three seasons. (29).

The drawbacks so far presented will only be exacerbated in the event of a pandemic. The current egg-based method of vaccine production cannot scale up rapidly enough to respond to a novel pandemic virus and vastly increased demand (30). In addition, viruses of avian origin can be highly virulent in chickens, reducing the ability to produce sufficient eggs for vaccine production (30). The shortcomings of egg based vaccines may soon be overcome by vaccines produced via tissue culture (30), as such a vaccine has been developed by Novartis (Hamburg, Germany) and was recently approved for use in the United States by the FDA (31). Even with

some of the logistical drawbacks, vaccines are still the most effective and reliable tool for the control and prevention of seasonal and pandemic influenza.

While preventative measures such as hygiene and vaccination are important in controlling the spread of influenza, they do nothing for an individual who is already infected. However, there are two different classes of antivirals that can be used to control an infection. These are the neuraminidase inhibitors and the adamantanes (32). Neuraminidase inhibitors including zanamivir and oseltamivir are substrate analogs of neuraminidase, a surface protein that cleaves sialic acid residues on the host cell membrane and is required for efficient virion release (33). By blocking neuraminidase activity, virions remain tethered to host cells following budding and therefore cannot infect new cells. The Centers for Disease Control (CDC) recommend treatment with zanamivir and oseltamivir for all cases of suspected or confirmed influenza (32). The drugs can also be used for chemoprophylaxis prior to or after a potential exposure (32). Two significant advantages of neuraminidase inhibitors are relatively low toxicity (few side effects), and their effectiveness against both influenza A and B (34). However, they should be used as soon as possible after symptom onset, as viral replication peaks in the lungs between 24 and 72 hours after symptoms appear (34).

The second class of antivirals, the adamantanes, includes amantadine and rimantadine (32). These work by blocking the M2 ion channel, preventing acidification of the capsid interior and subsequent dissociation of the capsid during endosomal escape (33). Like the neuraminidase inhibitors, the adamantanes can be used to treat influenza as well as to provide chemoprophylaxis before or after a potential exposure. Adamantanes also have a similar window of effectiveness, requiring that they be used within 24 hours of symptom onset. Unlike

the neuraminidase inhibitors, the adamantanes have more pronounced side effects and are only effective against influenza A viruses (34).

While antiviral drugs may be beneficial as treatment or prophylaxis for infection during flu season or a pandemic, their main drawback is the development of resistance. This is caused by mutations in neuraminidase or the M2 ion channel that reduce binding of their respective drugs (32). The potential for the loss of drug efficacy due to the development of resistance is best shown in the adamantanes. After two decades of use since their licensing in some countries, resistance to adamantanes had remained low, with rimantadine resistance found in only 0.8% of recovered isolates (35). Starting in 2003-04, an increasing percentage of influenza A strains demonstrated resistance to amantadine and rimantadine (32), causing the CDC to recommend discontinuation of their use in 2006 for the remainder of the 2005-06 flu season (36). Now, all currently circulating H3N2 strains, as well as the 2009 H1N1 virus are resistant to adamantanes (32), thus rendering them ineffective as an option for prophylaxis or treatment of influenza. In comparison, the neuraminidase inhibitors remain effective as few resistant isolates have been found thus far (32). However, similarly low levels of resistance to adamantanes had been seen after years of use. Therefore it is possible that widespread resistance to neuraminidase inhibitors may eventually develop, rendering the last currently available class of influenza antivirals useless.

RNA interference and the basics of RNAi therapeutics

An attractive alternative to antivirals is the development of treatments based on the mechanism of RNA interference. RNA interference was discovered by Dr. Craig Mello and Dr. Andrew Fire (37), who published their results in *Nature* in 1998 (37, 38). Since then, it has been utilized as an indispensable tool in molecular biological research, allowing for the study of gene function that

was only previously possible with the generation of knockout strains of organisms. By exploiting the RNAi pathway, one can study the effects resulting from the silencing of any gene of interest. In natural circumstances, the RNAi pathway begins when double stranded RNA is cleaved by Dicer, an RNAse III enzyme, to produce short interfering (si) RNA duplexes between 21 and 24 nucleotides in length with 2 nucleotide 3' overhangs (37). Dicer and the siRNAs then participate in the formation of the RNA Induced Silencing Complex (RISC) (39). While the comprehensive makeup of RISC is not entirely understood, the core consists of a member of the Argonaute family of proteins, which include Ago1-4 in humans (40). The RISC selects and binds the guide (antisense) strand of the duplex, making the distinction based on which 5' end has the lower melting temperature (41). The duplex is unraveled as Ago2 degrades the passenger (sense) strand, leaving the guide strand complexed with RISC (42). The loaded RISC complex binds mRNAs with sequences complementary to that of the guide strand, which are then degraded by the slicer activity of Ago2 (40). In experimental circumstances, chemically synthesized siRNAs are often used in place of those naturally generated from dsRNA via Dicer, yet the pathway forward is the same (41). As a finite quantity of siRNA is transfected into cells, this system is ideal for applications in which knockdown can be short-lived. For longer term knockdown, plasmids expressing short hairpin (sh) RNAs can be transfected into target cells. The continuously expressed shRNAs are shuttled out of the nucleus via Exportin-5 (43). and serve as substrates for Dicer in the cytosol (37), resulting in siRNA duplexes that enter the RNAi pathway as previously described.

Custom synthesis of oligonucleotides allows for the silencing of any gene of interest, so long as the sequence is known. This makes the RNAi pathway attractive to those designing novel biological therapeutics, as the transient alteration of gene expression can be used to treat a variety of illnesses ranging from macular degeneration (37) to cancer (39). In addition, RNA

interference has shown promise as a strategy in the development of antiviral drugs, as silencing viral genes can greatly hinder replication and thus control infection. This makes sense, as evidence suggests one of the evolutionary purposes of RNAi in eukaryotes was to protect against viruses by using dsRNA replication intermediates as substrates for Dicer (44). Antiviral strategies based on RNA interference have been investigated for many pathogens of public health significance, as well as some emerging viruses. It has been shown that siRNA and shRNA against the Hepatitis E RNA dependent RNA polymerase (RdRP) significantly inhibits HEV replication *in vivo* (45). A treatment for HIV-1 is currently under development that utilizes vectors that produce multiple shRNAs to hinder viral replication in CD4+ T cells (46). Gu et al. was able to show a reduction in cervical cancer tumor growth from human papillomavirus through the expression of shRNAs against viral E6 and E7 as well as angiogenesis factor VEGF (47). In 2003, Ge et al. demonstrated that targeting potent siRNAs to conserved influenza viral proteins significantly inhibited viral replication in cell culture and in embryonated chicken eggs (48). Rajput et al. later reduced influenza A replication in mice by administering siRNAs against NS1 (49), further demonstrating the potential for siRNAs as a clinical alternative to antiviral drugs for influenza. The particular strategy used depends on the nature of the virus. The transient gene knockdown from siRNA duplexes would be best suited for acute viral infections such as influenza, while chronic infections such as Hepatitis B would be better managed using vectors expressing shRNAs (50). RNA interference-based antivirals against Respiratory Syncytial Virus (RSV), Hepatitis C, and HIV are currently undergoing clinical trials (51), demonstrating that such therapeutic strategies could one day be reality.

One of the major advantages of RNAi based antivirals is that they utilize a mechanism present in nearly all eukaryotic cells (39). This means that viruses inhabiting any cell type can be targeted, which is especially useful for viruses such as filoviruses, which infect a variety of cell types (52).

Another major advantage is that any gene of the cell or virus can be exploited using the same mechanism, which allows for the inhibition of proteins for which small molecule inhibitors have not yet been discovered (53). RNAi treatments could therefore be optimized by selecting the most effective viral or host target to hinder viral replication. On the same vein, resistance to RNAi treatments could be countered by updating the siRNA sequences in response to escape mutants. In the case of influenza, siRNAs corresponding to the most recent seasonal isolates could be produced each year to keep up with antigenic drift. These aspects of RNAi give antiviral developers a wide range of flexibility in terms of drug design. In addition to its molecular advantages, RNAi therapeutics have logistical benefits as well. In comparison to biological therapeutics such as antibodies, chemically synthesized siRNAs are easier and cheaper to produce (53). Another logistical advantage over other biologics is that lyophilized siRNAs can be stored and transported at room temperature (53), reducing the need for a cold chain to transport the drugs from manufacture to points of delivery.

While therapeutic interventions based on RNA interference have significant advantages that make them attractive for clinical applications, there are some challenges that must be overcome for these treatments to be viable options for patients. The first challenge to the practicality of RNAi therapeutics is the reliable means of delivering siRNAs or shRNA producing vectors to cells. One research group developing RNAi based antivirals against parainfluenza virus has shown successful intranasal delivery of naked siRNA alone or complexed with a liposome transfection reagent in mice (54). However, this approach can lead to degradation of the siRNA if exposed to blood and other bodily fluids surrounding the target tissue (55). One way to get around this problem is to encase the siRNA or shRNA vector inside a viral transducing particle. One technique that has been commonly investigated for shRNA delivery is lentiviral transduction (43, 56). One benefit of this strategy is that the shRNA vector is integrated into the host genome for

continued expression (56), which could be advantageous for treatment of a chronic viral infection such as HIV or Hepatitis C, or for prophylaxis against these or other viruses. For siRNA duplexes, it has been shown that reconstituted influenza virus membranes can be used to form virus like transducing particles in conjunction with cationic lipid complexes to aid transfection (55). In addition to protecting nucleic acid payloads *in vivo*, viral transduction particles have the added advantage of being readily made in the laboratory (43, 55, 56) and their tissue tropism can be modified via appropriate pseudotyping of surface proteins (56).

While sequence specificity confers some of the advantages of RNAi therapeutics, it also provides the drawback of increased drug resistance. As siRNAs require high sequence homology with their target along their 19-21 nucleotide stretch for adequate gene silencing, they may be less effective against highly mutable viruses such as influenza and HIV (53). However, resistance can be countered by updating the target sequence to incorporate the mutation, target a different sequence in the gene, or use a pool of siRNAs to target sequences on the same or multiple genes (53). In addition, loss of efficacy to developing resistance can be mitigated by targeting sequences that are known to be conserved (53).

Another challenge impeding the use of RNAi based therapeutics is the potential for off-target effects. One source of off-target effects is partial pairing of the siRNA guide strand with mRNAs from genes besides the intended target (39). It has been found that as few as 11 matching nucleotides between the guide strand and mRNA can be enough for off-target degradation, while as few as 6 or 7 matching nucleotides can cause degradation if located in the 'seed region' near the 5' end of the guide strand (39). The degradation of non-target mRNAs can be deleterious if they encode gene products crucial to cell viability and survival. Such off-target effects present a serious drawback that must be overcome if RNAi based therapies will ever see

widespread use in a clinical setting. Another source of off-target effects is through elicitation of innate immune responses in the cell. As the majority of RNAi mechanisms involve double stranded RNA, there is the potential for activation of innate interferon responses via double stranded RNA-activated protein kinase (PKR) and toll-like receptors against single and double stranded RNA (39). While this is more of a problem for RNAi therapies involving long dsRNAs, it has been shown that siRNAs as short as 23 nucleotides can elicit an interferon response (57). The triggering of innate immune responses by siRNAs has been shown to be sequence dependent, with GC rich siRNAs posing the highest risk (57). Along with off-target effects, another major problem in RNAi therapeutics is achieving the optimal dose. The treatment may be ineffective if too little siRNA is delivered to target cells, and delivery of too much siRNA increases the likelihood of off-target effects (53).

Theory behind the development of pro-siRNAs

Described herein, is a project that was conducted in a collaborative effort between the laboratory of Dr. Andrew Pekosz and the Johns Hopkins Applied Physics Laboratories to develop a novel RNAi based therapeutic strategy against influenza. Our approach utilizes a pro-siRNA design concept that exploits the cap-snatching activity influenza must utilize to complete its life cycle. As was previously mentioned, the PA endonuclease cleaves host pre-mRNAs 10-13 nucleotides downstream of their caps, using this capped extension as a primer for the transcription of its own genes. Therefore, our pro-siRNA design will consist of a traditional siRNA duplex plus a 10-13 nucleotide extension on the 5' end of the sense strand, terminating in a standard 7-methylguanosine cap (Figure 1). In theory, this extension should render the pro-siRNA inert in uninfected cells. When cells are infected, the capped extension is cleaved via influenza PA endonuclease activity, leaving behind a normal duplex. The activated siRNA

duplexes can then enter the RNAi pathway, targeting viral proteins such as NP, the M2 ion channel, or the polymerase PB1, any of which would hinder replication. An influenza activated pro-siRNA would solve two of the major problems with RNAi therapeutics. By requiring virus for activation, off-target effects from pro-siRNAs would be limited to infected cells, sparing healthy tissues. In addition, the optimal delivery dose of the pro-siRNA would be more flexible than conventional siRNA because the active dose is modulated by viral activity. More importantly, a pro-siRNA that is only activated in infected cells could be used to target host proteins such as the anti-apoptotic factor Bcl-2 (58) to alter the outcome of infection. Host genes are far less mutable than viral genes and could make for superior targets to knock down viral replication, but could not be safely exploited with conventional siRNAs.

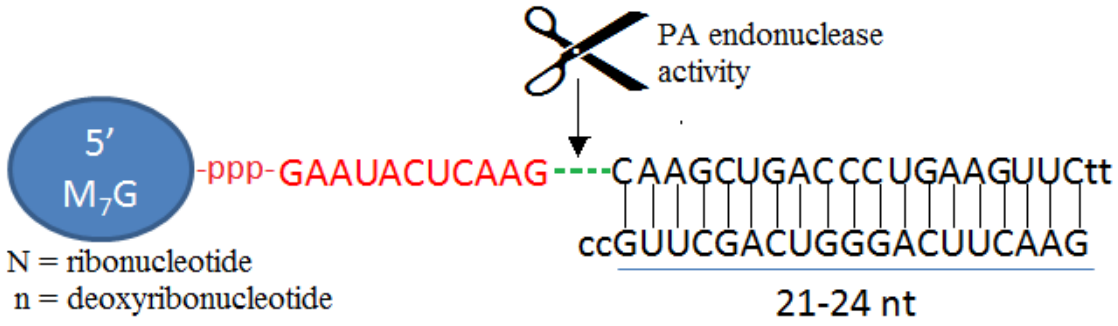

Figure 1: The basic design of a pro-siRNA against eGFP, consisting of an siRNA duplex of functional length (21-24 complementary nucleotides) with a capped 5' extension on the sense strand (red letters). The pro-siRNA construct should be inert until the 5' extension is cleaved by the endonuclease activity of PA, a component of the heterotrimeric influenza polymerase complex. The remaining duplex should then be able to enter the RNAi pathway and silence eGFP expression. This approach could be used to silence viral or host proteins to alter the outcome of infection.

While, our pro-siRNAs will be designed and tested with influenza, the same concept can be applied to other viruses that utilize cap snatching in their life cycles, such as Bunyaviruses (7). The Bunyaviridae family contains the Hantaviruses, known for causing Hantavirus Pulmonary

Syndrome (HPS), a severe and potentially fatal condition in which the lungs fill with fluid (59). By the end of 2012, a total of 613 cases of HPS were reported in the United States, of which 33% were fatal (59). If successful, the pro-siRNA concept could be applied to potentially lifesaving treatment of Hantavirus infections, for which there are no antivirals currently available (59). While pro-siRNAs are designed for use with cap snatching viruses, other RNAi delivery strategies could be developed to exploit key life cycle steps in other viruses, such as shRNAs produced from a gene cassette regulated by a viral promoter.

The focus of this research is to investigate the feasibility of the pro-siRNA concept in which exogenously delivered pro-siRNAs can knockdown a gene of interest in an infected cell while having minimal effect on said gene in a cell not infected with influenza virus. This will require the development of experimental protocols to test for gene knockdown, as well as careful design and development of the pro-siRNA constructs, including optimization of their *in vitro* assembly.

Materials and Methods

REAGENTS

Plasmid DNA:

The pHH21 NP UTR Hi eGFP plasmid produces eGFP in an infection dependent manner by expressing antisense eGFP transcripts that resemble influenza gene segments transcribed by viral polymerase. Construction and testing of this plasmid has been previously described (60). pCAGGSeGFP produces eGFP continuously via the chick β-actin fibroblast promoter. pCAGGSeGFP and pHH21 NP UTR Hi eGFP were transformed into competent *E. coli DH5α* cells. Selection for transformed cells was performed by spreading onto an LB agar plate containing 100 µg/mL ampicillin and incubating at 37°C for 16-18 hours. Plasmids were purified from *E. coli* using a MaxiPrep kit (QIAGEN) as per the manufacturer's instructions. Plasmid concentration was measured using a Nanodrop spectrophotometer.

siRNA:

SiRNAs against eGFP were acquired from Ambion (Life Technologies) with the following sequences: 5' CAAGCUGACCCUGAAGUUCdTdT 3'(sense)and 5'GAACUUCAGGGUCAGCUUGdCdC 3'(antisense). Scrambled siRNAs (Negative Control #1) were also obtained from Ambion with the following sequences: 5' AGUACUGCUUACGAUACGGdTdT 3' (sense) and 5'CCGUAUCGUAAGCAGUACUdTdT 3' (antisense).

Pro-siRNA construction:

Sense and antisense strands of the pro-siRNA constructs and controls were purchased from FidelitySystems Inc. The triphosphorylated sense strands were capped using the CellScript M_7G

capping system (Cellscript) as per the instructions of the manufacturer, except for capping 200 pmol of oligo per reaction and allowing the reaction to proceed at 37°C for 1 hour instead of 30 minutes. The capped sense strands were purified using RNA Clean and Concentrator columns (Zymo Research Corporation) as per the instructions of the manufacturer. The sense and antisense strands were combined in equimolar amounts in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM magnesium acetate), placed in a thermocycler preheated to 90°C and heated for 1 minute at 90°C followed by 1 hour at 37°C. Annealing was verified by running 10 pmol of annealed siRNA on a 12% native polyacrylamide gel. Bands were stained for 5 minutes using a 1:20,000 dilution of ethidium bromide (stock is 10 mg/mL) in 0.5x TBE buffer and visualized under a UV transilluminator.

CELL CULTURE AND TRANSFECTIONS

Cell Lines:

A549s were maintained in DMEM with 10% FBS, 1% Penicillin and Streptomycin and 1% L-Glutamine at 37°C with 5% CO_2, and were passaged 1:8 when reaching 80-90% confluence (every 3-4 days). For liposome transfections, approximatey 2.8x10^5 cells were plated in each well of a six well plate 24-26 hours prior to transfection to target 60-80% confluence at the time of transfection.

293Ts were maintained in DMEM with 10% FBS, 1% Penicillin and Streptomycin and 1% L-Glutamine at 37°C with 5% CO_2, passaged 1:10 every three days. Six well plates coated with poly-L-lysine were seeded with approximately 1.2x10^6 cells per well 16-24 hours prior to transfection to target 70-90% confluence at the time of transfection.

293Ts stably transfected with pHH21 NP UTR eGFP were maintained similarly as above, except for the addition of Hygromycin B (Roche) to the media at a final concentration of 250 μg/mL to select for cells carrying the plasmid. These cells were passaged 1:10 every four or five days. Unless indicated, six well plates coated with poly-L-lysine were seeded with approximately 6 x 10^5 cells per well 36-48 hours prior to transfection.

Madin Darby canine kidney (MDCK) cells stably transfected with the pCAGGSeGFP plasmid were maintained in DMEM with 10% FBS, 1% Penicillin and Streptomycin and 1% L-Glutamine at 37°C with 5% CO_2 and were passaged 1:10 every 2-3 days. The pCAGGSeGFP plasmid was maintained through addition of puromycin to a final concentration of 7.5 μg/mL every other passage. Transfection of MDCKpcGFP cells was performed in suspension, seeding approximately 1.5x10^5 cells per well on 12 well plates at the time of transfection.

Liposome Transfections:

DNA and siRNA liposome cotransfections were carried out using Lipofectamine 2000 (Life Technologies) as follows. Lipofectamine 2000 (4 μL per well for 293T and A549, and 6 μL per well for MDCK) and nucleic acids (1 μg plasmid DNA and/or 30 pmol siRNA per well) were diluted in separate tubes of OPTIMEM (50 μL per well) and allowed to incubate for 5 minutes. Equivalent amounts of the diluted lipofectamine were added to each tube of diluted plasmid DNA and siRNA, gently mixed and incubated for 20 minutes to facilitate complex formation. The complete DMEM in each well was replaced with 1mL OPTIMEM before 105 μL of the transfection mixtures were added (final transfection volume is 1.1 mL). Cells were allowed to incubate with the transfection complexes at 37°C for 4 hours before 1 mL complete media was added to each well. Mock transfections were performed using this same procedure, except that

no transfection mixture was ever applied to the cells, meaning they never received lipofectamine 2000 nor plasmid DNA or siRNAs.

Nucleofection:

A549s were subcultured by seeding 3 x 10^6 cells into one or more T150 flasks two days prior to transfection, targeting 70-80% confluence on the day of transfection. Nucleofection was performed using the Amaxa Nucleofector II device with the Nucleofector Solution T kit as per the optimized protocol for A549 cells provided by the manufacturer, using 2 μg of pCAGGSeGFP plasmid and 30 pmol of siRNA per nucleofected sample. For virus-free experiments, the contents of each cuvette were distributed dropwise evenly across a single well of a six well plate using a sterile cell dropper provided with the kit. For experiments comparing infection with mock infection, the cell suspension from each cuvette was divided across two separate wells on a six well plate (approximately 5x10^5 cells per well).

INFECTION

Infection Media was prepared with DMEM with 1% Penicillin and Streptomycin, 1% L-Glutamine and 0.5% Bovine Serum Albumin (BSA). Infection was performed 24-26 hours post transfection. Plates were washed twice with GIBCO Dulbecco's PBS+ (with calcium and magnesium, Life Technologies) to remove all traces of serum. For 293Ts and A549s on 6 well plates 250 μL of inoculum was added to infected wells while 250 μL of infection media was added to mock infected wells. For MDCK cells on 12 well plates, 200 μL of either inoculum or infectious media was used. Cells were infected with a multiplicity of infection (MOI) of 5 infectious viruses per cell to ensure 70-90% of cells are infected with only one round of replication. The plates were placed on a rocker at room temperature for 1 hour to attach viruses to the cell surfaces. Excess

inoculum was then removed, followed by washing with PBS+ and the addition of 1 mL of infectious media per well. The plates were then placed in a 37°C incubator to initiate infection.

FLOW CYTOMETRY

For virus-free experiments, cells were harvested at 24 hours post transfection. Otherwise they were harvested 24 hours post infection. Harvesting was performed by washing the plates with Dulbecco's PBS followed by the addition of a 1:5 dilution of 0.5% Trypsin-EDTA solution in PBS. Cells were allowed to incubate until detachment. The trypsinized cells were fixed in suspension with equal volume 4% paraformaldehyde in Dulbecco's PBS. Cells were then washed twice with Dulbecco's PBS. To identify infected cells, cells were permeabilized with 0.2% Triton X100 in PBS and allowed to incubate for 10 minutes, then washed twice with PBS. Cells were then mixed with blocking solution (3% normal goat serum, 0.5% BSA in PBS) and allowed to incubate for 1 hour, followed by incubation in mouse anti-NS1 (1:100) solution for 1 hour. The cells were then washed three times with PBS and allowed to incubate with goat anti-mouse antibodies (1:500) for 1 hour. The goat anti-mouse antibodies are conjugated to Alexafluor 647 to aid detection by flow cytometry.

For each experiment, the Becton Dickinson FACSCalibur was set to read eGFP fluorescence via the FL1 channel, and infection (Alexafluor 647) via the FL4 channel. Both channels were set to record fluorescence on a logarithmic scale. Ten-thousand cells are counted in each sample.

Flow cytometry results were analyzed using FlowJo 7.6. Gating on the FL1 channel was used to delineate GFP positive populations from GFP negative populations, as well as infected populations from uninfected populations. The geometric mean fluorescence intensity was calculated for GFP positive populations. Results of the analysis were imported into GraphPad Prism 4.0. Normalization was conducted by assigning the value of 100% to the average of the

scrambled siRNA replicates in an experiment, then calculating the percentage of each data point relative to that average.

STATISTICAL ANALYSIS

Statistical analysis was performed using the statistics package included in the GraphPad Prism 4.0 software. A one-way analysis of variance (ANOVA) was used to test for differences between multiple conditions, followed by a Bonferroni post-test to compare the differences between conditions individually. A p-value less than 0.05 ($P<0.05$) is considered to be statistically significant.

CHAPTER 2: Development of Experimental Procedures

INTRODUCTION

To test concept of our pro-siRNA design, we will utilize enhanced green fluorescent protein (eGFP) as our target. The advantage of using eGFP in a proof of principle study is that eGFP expression can be readily measured by flow cytometry without using immunofluorescence. In addition, eGFP plasmids are commonly used transfection controls in molecular biology and siRNAs targeting eGFP are common positive controls in the establishment of knockdown in RNAi experiments. Therefore, eGFP is the most suitable target for a proof of principle experiment involving novel pro-siRNA designs, as knockdown can clearly be identified.

Before testing the pro-siRNA concept, one must first develop the methods to screen design candidates. To do this, one can transfect cells with an eGFP expressing plasmid and conventional eGFP siRNAs to determine the appropriate conditions for transfection, the amount of siRNA required for knockdown and under what timetables knockdown can be observed. It is also important to examine whether or not infection interferes with knockdown of eGFP expression, as that could confound the results of experiments testing the effect of the pro-siRNAs, which are activated by viral activity. Once these parameters are determined, one can then use the conventional eGFP siRNAs as a positive control with which to compare the effect of the pro-siRNAs on eGFP expression.

Another variable that must be determined is which cell type should be used to test for eGFP silencing activity by pro-siRNAs. Options under consideration include Madin Darby canine kidney (MDCK) cells stably expressing eGFP, human embryonic kidney 293T cells either transiently transfected with a continuous eGFP expression plasmid or stably transfected with a plasmid that require infection for eGFP expression, and A549 (human alveolar carcinoma) cells

transfected for continuous eGFP expression. The advantages and disadvantages of each possible experimental system must be determined in terms of relevance, ease of transfection and demonstration of adequate eGFP silencing effect. We did this by establishing successful knockdown of eGFP expression in each of these cell types, and demonstrating that influenza virus infection did not significantly alter silencing in any of the developed experimental systems. Establishing procedures to demonstrate reproducible knockdown of eGFP expression in several cell types gives multiple options with which to test the pro-siRNA concept.

In addition to developing the appropriate assay systems, one must also determine the best means of measuring and quantifying reductions in eGFP expression. Two possible metrics include the percentage of GFP positive cells and the mean fluorescence intensity (MFI) of eGFP expressing cells. While the former can be affected by the administration of eGFP siRNAs, the value of percent positive cells is largely dependent on gating, which is difficult to standardize from experiment to experiment. Different cell types express eGFP differently and thus have differently shaped histograms. This necessitates different gating strategies for each cell type to accurately delineate the eGFP positive population. This dramatically affects the difference in percent eGFP positive cells that can be seen in siRNA knockdown experiments, with some cell types showing a dramatic reduction in eGFP positive cells, while another may show little reduction at all.

On the other hand, the mean fluorescence intensity is a measure of the average brightness of each cell, indicative of the eGFP expression in that particular cell. This metric is superior to the percent positive eGFP expressing cells for two reasons. The first reason is that it takes into account the realities that eGFP expression will not be silenced completely in a transfected cell, and that not all cells will be transfected with eGFP siRNAs. On the contrary, measuring the

reduction in eGFP expressing cells assumes that eGFP expression is completely silenced in transfected cells. More importantly, the second reason is that the measurement of the MFI is less dependent on gating. While the value of the MFI can depend on the range of the gate, the difference between the MFIs of cells receiving eGFP siRNA versus scrambled siRNA is less affected by variances in gating between cell types. While some cell types can vary widely in the observed decrease in the percentage of eGFP positive cells from 0-70%, these same cell types display a more similar reduction in the MFI of eGFP positive cells, ranging from 40-60%. For these reasons we measured siRNA mediated knockdown of eGFP expression by comparing conditions by the mean fluorescence intensity of eGFP expressing cells.

RESULTS

To validate the eGFP siRNAs from Ambion (Life Technologies), we tested them in Madin-Darby canine kidney (MDCK) cells that were stably transfected with a plasmid that expresses eGFP. This cell line was chosen for the first experiments because the stable expression of eGFP eliminated the need to transfect an eGFP expressing plasmid in addition to the eGFP siRNAs, eliminating a potentially complicating variable. In these experiments, MDCK pCAGGseGFP cells were transfected with 30 pmol of either eGFP siRNAs or scrambled siRNAs with no known target sequence. To control for potential silencing effects of the scrambled siRNA, a subset of cells were also mock transfected. After 24 hours, the cells were harvested and fixed, and fluorescence indicating eGFP expression was measured by flow cytometry. MCDK pCAGGseGFP cells transfected with eGFP siRNAs showed a significant reduction in eGFP expression as compared to cells transfected with the scrambled siRNAs, as indicated by a twofold decrease in mean fluorescence intensity (Figure 2a). This effectively demonstrated that the eGFP siRNAs from Ambion would suffice for our purposes.

The next important step was to determine whether or not infection would impede the previously observed knockdown of eGFP expression. If that were the case, one could not accurately determine the knockdown effect of the pro-siRNAs as the effect of virus infection would compound the results. To test for possible effects of virus infection on knockdown, MDCK pCAGGSeGFP cells were transfected as above. At 24 hours post transfection, cells were either mock infected or infected with rUdorn virus ensuring approximately five infectious viruses per cell. Such a high multiplicity of infection is used so that between 70% and 90% of cells will be infected without subsequent rounds of infection. Cells were harvested and fixed 24 hours post infection and viral NS1 was detected by immunofluorescence to identify infected

populations of cells. Fluorescence from eGFP expression and NS1 was measured by flow cytometry. Infection was not impeded by the liposome mediated transfection, as indicated by

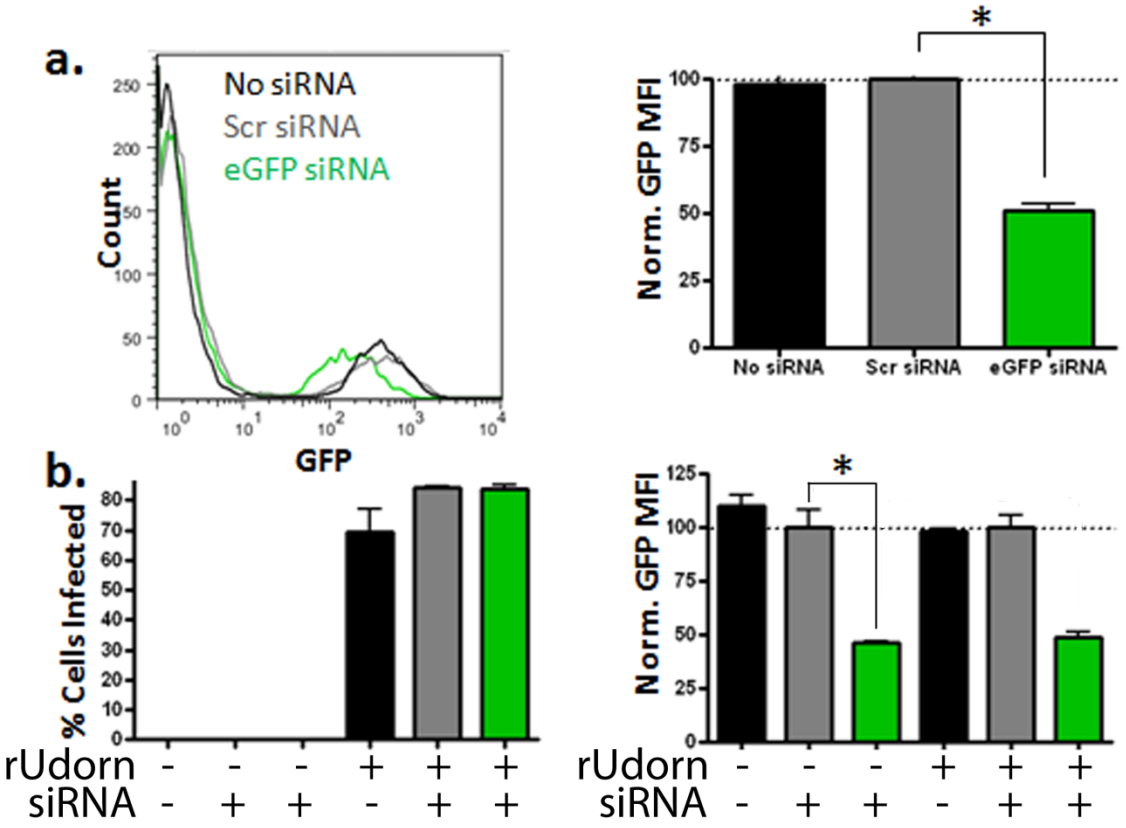

Figure 2: The Silencer eGFP siRNAs (Life Technologies) were validated using MDCK cells stably transfected with pcGFP plasmid, which continuously expresses eGFP. **a.** MDCKpcGFP cells were transfected with eGFP siRNAs (G) or scrambled siRNAs (S). The stably expressing cells were mock transfected to control for possible effect of the scrambled siRNA on eGFP expression. Cells were harvested and fixed 24 hours post transfection and fluorescence indicating eGFP expression was measured by flow cytometry. The mean fluorescence intensity shown is relative to scrambled siRNA, which is normalized to 100%. Cells transfected with eGFP siRNAs showed a significant reduction in mean fluorescence intensity as compared with cells transfected with scrambled siRNA. **b.** MDCKpcGFP cells were transfected as above. Cells were either mock infected or infected with rUdorn virus (MOI=5) 24 hours post transfection. Cells were fixed 24 hours post infection. Infected populations were identified by immunofluorescence for viral NS1 protein. Fluorescence indicating eGFP expression and infection was measured by flow cytometry. Statistical significance ($P<0.05$) is indicated by a star (*).

the achievement of at least 60% of cells infected in both mock transfected cells and cells transfected with siRNAs (Figure 2b). As found previously, MDCK pCAGGseGFP cells transfected with eGFP siRNAs showed a roughly twofold decrease in mean fluorescence intensity (MFI) as

compared to cells transfected with scrambled siRNAs, indicating a significant reduction in eGFP expression in eGFP siRNA transfected cells (Figure 2b). However, there was no significant difference in the MFI of infected cells and uninfected cells receiving eGFP siRNAs as compared with infected and uninfected cells transfected with scrambled siRNAs respectively (Figure 2b), indicating that virus infection does not impede with the knockdown of eGFP siRNAs. The above results indicate that transfection of MDCKpCAGGseGFP cells could be a suitable means of testing pro-siRNA designs for knockdown activity.

While an experiment system using the MDCK pCAGGseGFP cells would work well as a pro-siRNA screening method, it must be noted that these are not human cells. The pro-siRNA treatment would eventually be performed in human patients and would therefore need to be validated using a human cell line. A step in the right direction would be the use of immortalized human embryonic kidney (HEK293T) cells. As a human cell line, 293T cells are a more relevant cell type than dog kidney cells with respect to the eventual application of pro-siRNAs against influenza. The cells we used were not stably transfected with a continuous eGFP producing plasmid, requiring transient transfection in order to produce eGFP during the duration of an experiment. Fortunately, Lipofectamine 2000 can cotransfect plasmid DNA along with siRNAs simultaneously resulting in little change to the procedure (61). For simplicity, we used the pCAGGSeGFP plasmid that had been stably transfected into the MDCK cells used in the previous experiments. To evaluate the effect of eGFP siRNAs on transient eGFP expression, 293Ts were cotransfected with 1 µg pCAGGSeGFP and 30 pmol of either eGFP siRNAs or scrambled siRNAs. To evaluate the potential silencing effects of the scrambled siRNAs, a subset of the 293Ts were transfected with pCAGGSeGFP alone. The cells were harvested and fixed 26 hours post transfection, and fluorescence from eGFP expression was measured by flow cytometry. Transfection of 293Ts with eGFP siRNAs along with pCAGGSeGFP resulted in a significant reduction in eGFP expression

as compared with 293Ts cotransfected with pCAGGSeGFP and scrambled siRNAs, as indicated by an over 60% (62-65%) reduction in mean fluorescence intensity (MFI) of eGFP positive cells (Figure 3a).

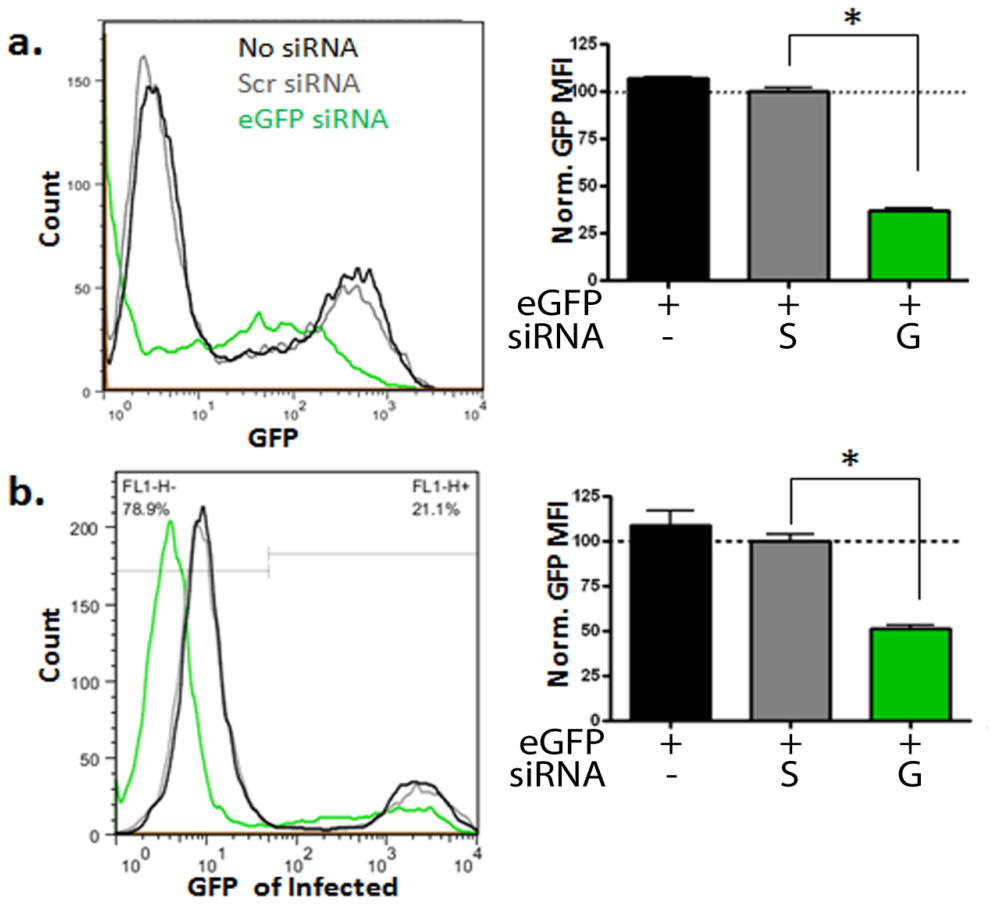

Figure 3: a. 293Ts were cotransfected with pCAGGSeGFP (eGFP) plasmid and either eGFP siRNAs (G) or scrambled siRNAs (S). Cells were transfected with pCAGGSeGFP alone to control for possible effect of the scrambled siRNA on eGFP expression. Cells were harvested and fixed 26 hours post transfection and fluorescence indicating eGFP expression was measured by flow cytometry. The mean fluorescence intensity shown is relative to scrambled siRNA, which is normalized to 100%. Cells cotransfected with pCAGGSeGFP and eGFP siRNAs showed a significant reduction in mean fluorescence intensity compared to cells cotransfected with pCAGGSeGFP and scrambled siRNAs. **b.** 293Ts stably carrying the VIRGS plasmid were transfected with 30 pmol of scrambled or eGFP siRNAs. Cells were infected 24 hours post transfection and fixed 24 hours post infection. Infected populations were identified by immunofluorescence for viral NS1 protein. Fluorescence indicating eGFP expression and infection was measured by flow cytometry. The eGFP fluorescence and mean fluorescence intensity (MFI) measures of the infected population are shown. Statistical significance ($P<0.05$) is indicated by a star (*).

In addition to transient transfection of 293Ts with pCAGGSeGFP, we also utilized 293Ts that were stably transfected with pHH21 NP UTR Hi eGFP, also known as the Virus Induced Reporter Gene Segments (VIRGS) plasmid. The VIRGS plasmid expresses negative sense transcripts of eGFP flanked by the 5' and 3' untranslated regions of the A/WSN/1933 nucleoprotein gene, resulting in RNAs that resemble influenza genomic RNA (60). These are transcribed by the polymerase complex to produce mRNAs that can be translated by host ribosomes to produce eGFP. Therefore, eGFP is only produced in VIRGS transfected cells when they are infected. Such a system would be useful when trying to evaluate eGFP knockdown mediated by the virus activated pro-siRNAs, as only infected cells produce eGFP and the eGFP expression begins at the same time that the pro-siRNAs are activated, potentially giving cleaner results. In these experiments, 293T VIRGS cells were transfected with 30 pmol of siRNAs targeting eGFP or scrambled siRNAs with no known target sequence. At 24 hours post transfection, cells were infected with rUdorn virus at a multiplicity of infection (MOI) of 5 infectious viruses per cell. Cells were fixed and harvested 24 hours post infection and cells containing viral NS1 protein were identified by immunofluorescence. Fluorescence from eGFP expression or infection was measured by flow cytometry. 293Ts transfected with eGFP siRNAs showed a significant decrease in eGFP expression as compared with cells transfected with scrambled siRNAs as indicated by a nearly 50% (44-51%) reduction in the MFI of eGFP positive cells (Figure 3b). While not as dramatic as the knockdown observed in the pCAGGSeGFP experiments (Figure 3a), it is still suitable for screening pro-siRNA designs.

While it was previously found that infection with influenza virus did not impede siRNA mediated knockdown of eGFP expression in MDCK cells (Figure 2b), we needed to investigate whether or not this was also true in 293Ts. To answer this question, 293Ts were cotransfected with pCAGGSeGFP and siRNAs as before (Figure 3a). At 24 hours post transfection, the cells were

either mock infected, or infected with rUdorn virus at an MOI of 5 infectious viruses per cell. Cells were then fixed and harvested 24 hours post infection, and viral NS1 protein was identified by immunofluorescence to denote infected cells. Fluorescence from eGFP expression and NS1 was measured by flow cytometry. Infection was not impeded by liposome transfection in 293Ts as indicated by the achievement of 70% of cells infected in both transfected and mock transfected conditions using the same inoculum. (Figure 4b)

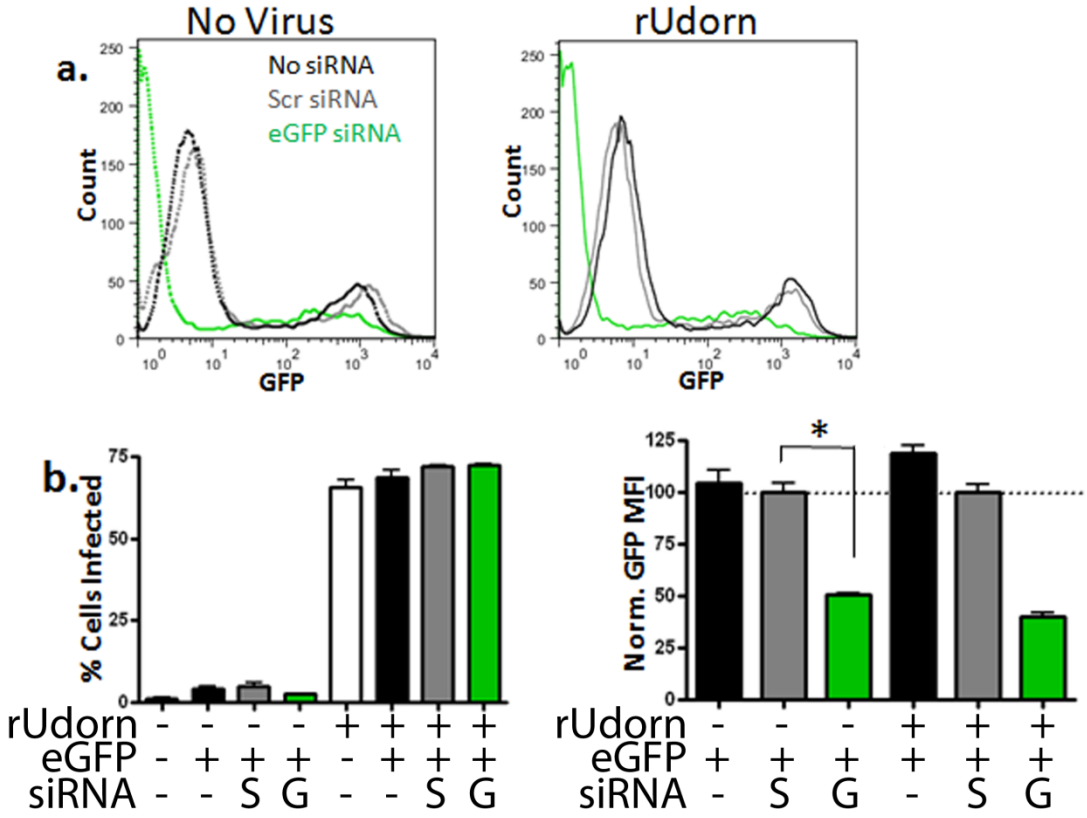

Figure 4: 293Ts were cotransfected with pCAGGSeGFP plasmid and eGFP siRNA (G) or a corresponding scrambled siRNA (S). Cells were also transfected with pCAGGSeGFP alone to control for possible effect of the scrambled siRNA on eGFP expression. Cells were infected 24 hours after transfection and fixed 24 hours post infection. Infected populations were identified by immunofluorescence for viral NS1 protein. Fluorescence indicating eGFP expression and infection was measured by flow cytometry. The mean fluorescence intensity shown is relative to scrambled siRNA, which is normalized to 100%. Statistical significance ($P<0.05$) is indicated by a star (*).

As was found in the previous pCAGGSeGFP cotransfection experiments (Figure 3a), cells receiving eGFP siRNAs showed reduced eGFP expression in comparison to cells receiving scrambled siRNAs with pCAGGSeGFP, as indicated by a reduction in overall fluorescence (Figure 4a) and a twofold decrease in the MFI of eGFP positive cells (Figure 4b). Like in MDCK pCAGGseGFP cells, infected and uninfected 293T cells cotransfected with pCAGGSeGFP and eGFP siRNAs did not show a significant difference in the reduction of overall fluorescence (Figure 4a) or MFI (Figure 4b) as compared with their respective cells receiving scrambled siRNA, indicating that virus infection does not impede eGFP knockdown in 293T cells. Since knockdown of eGFP expression can be readily observed in 293Ts that are cotransfected with a plasmid and siRNAs, and that knockdown is not impeded by infection, it is evident that this experimental system can reliably be used to screen pro-siRNA activity.

While the HEK293T cell line is an improvement over the use of MDCK cells, they are still kidney cells. A better cell line to use would be one that represents the human lung, as that is where influenza infection takes place and where a pro-siRNA treatment would be administered. We therefore investigated the use of the A549 human alveolar carcinoma cell line as a means of screening pro-siRNAs. Like the 293T cells, these were not stably transfected with a continuous eGFP producing plasmid, requiring cotransfection for siRNA experiments. First, we sought to determine what eGFP knockdown might look like in A549s. In these experiments, A549s were cotransfected with 1 µg of pCAGGSeGFP plasmid and 30 pmol of eGFP siRNAs or scrambled siRNAs. To evaluate possible silencing effects of the scrambled siRNA, a subset of cells was transfected with pCAGGSeGFP alone. The cells were harvested and fixed 26 hours post transfection and eGFP fluorescence was assayed by flow cytometry. It was found that cells receiving eGFP siRNAs showed a significant reduction in eGFP expression in comparison to cells

receiving the scrambled siRNAs along with pCAGGSeGFP as indicated by over 60% (61-65%) decrease in the MFI of eGFP expressing cells (Figure 5a).

While the results of the previous A549 experiments were promising, it was later discovered that there was a significant problem with this cell type. In subsequent experiments using the VIRGS plasmid, it was found

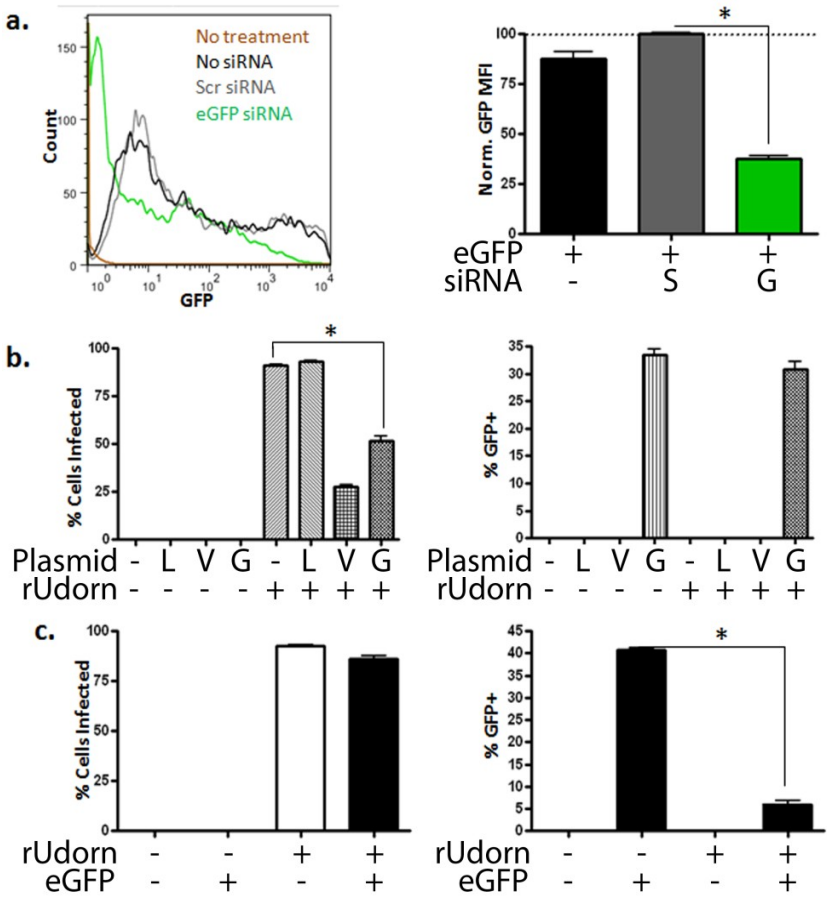

Figure 5: a. A549s were cotransfected with pCAGGSeGFP plasmid and either eGFP siRNAs (G) or scrambled siRNAs (S). Cells were fixed 26 hours post transfection and fluorescence indicating eGFP expression was measured by flow cytometry. The mean fluorescence intensity shown is relative to scrambled siRNA, which is normalized to 100%. Cells cotransfected with pCAGGSeGFP and eGFP siRNAs showed a significant reduction in mean fluorescence intensity compared to cells cotransfected with pCAGGSeGFP and scrambled siRNAs. **b.** A549s were transfected with pCAGGSeGFP (G) pCAGGS vector plasmid (V) or Lipofectamine 2000 with no plasmid (L). Cells were infected with rUdorn (or mock) 24 hours post transfection and fixed 24 hours post infection. Infected populations were identified by immunofluorescence for viral NS1 protein. Fluorescence indicating eGFP expression and infection was measured by flow cytometry. Statistical significance ($P<0.05$) is indicated by a star (*).

that infection was heavily impaired in transfected cells. This presents a serious problem if A549s were to be used to screen for pro-siRNA activity against eGFP, as they should require infection

to be active. To investigate this inhibition of infection, a series of troubleshooting experiments was conducted, one of which is chronicled below. In this experiment, A549s were transfected with the pCAGGSeGFP plasmid, the empty pCAGGS vector, Lipofectamine alone with no plasmid, or mock transfected. At 24 hours post transfection, cells were mock infected or infected with rUdorn virus at an MOI of 5 infectious viruses per cell. The cells were harvested and fixed 24 hours post infection, and infected cells were identified by immunofluorescence for viral NS1 protein. Fluorescence from infection and eGFP expression was measured by flow cytometry. As was seen before, the transfected A549s appeared to be refractory to infection, with cells transfected with both the vector plasmid and the pCAGGSeGFP plasmid producing significantly lower percentages of infection than the mock transfected control (Figure 5b). As both an expressing plasmid and a non-expressing plasmid produced a similar effect on infection, it shows that the act of transfection, not expression of the plasmid is likely responsible. It is also important to note that cells receiving Lipofectamine2000 without plasmids did not show a significant reduction in percentage of infection (Figure 5b), suggesting that complete DNA-lipofectamine transfection complexes might be the impeding factor.

If liposome transfection might be impeding infection in A549s, one could posit that infecting the cells first might alleviate the problem. This would make sense, as this would allow virus entry before possible interference from liposome-DNA complexes. In an experiment to investigate this possibility, A549s were either mock infected or infected with rUdorn virus at an MOI of 5 infectious viruses per cell, followed by mock transfection or transfection with 1 μg pCAGGSeGFP plasmid 1 hour later. The cells were harvested and fixed 24 hours post infection and infected cells identified by immunofluorescence for viral NS1. Fluorescence from infection and eGFP expression was measured by flow cytometry. While there was still a slight reduction in the percentage of transfected A549s infected in comparison to mock transfected cells (Figure 5c),

the decrease was not nearly as dramatic as was seen in the previous experiments in which A549s were transfected 24 hours before infection (Figure 5b). However, transfected cells that were previously infected showed a dramatic reduction in the percentage expressing eGFP compared to transfected cells that were previously mock infected (Figure 5c). This suggests that infection impedes either transfection or eGFP expression in A549s. Therefore, while infection prior to transfection alleviates the inhibition of infection by liposome-DNA complexes, the major interference with eGFP expression nullifies this experimental strategy as a solution to the problem.

If liposome mediated transfection impedes infection in A549s, another potential solution is to use a different transfection method. In this case, we explored nucleofection, an electroporation based technology from Amaxa (Lonza). Rather than using liposome-DNA complexes, nucleofection works through the combination of a proprietary solution in combination with a pulse of electric current to drive plasmid DNA and siRNA into the cytosol and nucleus simultaneously (62). To evaluate nucleofection as a transfection method for A549s, cells were transfected with 2 μg of pCAGGSeGFP and 30 pmol of eGFP siRNAs and scrambled siRNAs. A subset of cells was transfected with pCAGGSeGFP alone to monitor for potential silencing effect of the scrambled siRNA. At 24 hours post nucleofection, the cells were harvested and fixed and eGFP fluorescence was measured by flow cytometry. As was previously seen in the cotransfection of A549s with lipofectamine2000, cells receiving eGFP siRNAs showed a significant reduction in eGFP expression in comparison to cells receiving scrambled siRNAs along with the pCAGGSeGFP plasmid, as indicated by a nearly twofold decrease in MFI of eGFP positive cells (Figure 6).

While nucleofection was shown to be an effective alternative method for cotransfection of A549s, we needed to determine whether or not the procedure would impede infection as had been seen with the liposome transfections in that cell type.

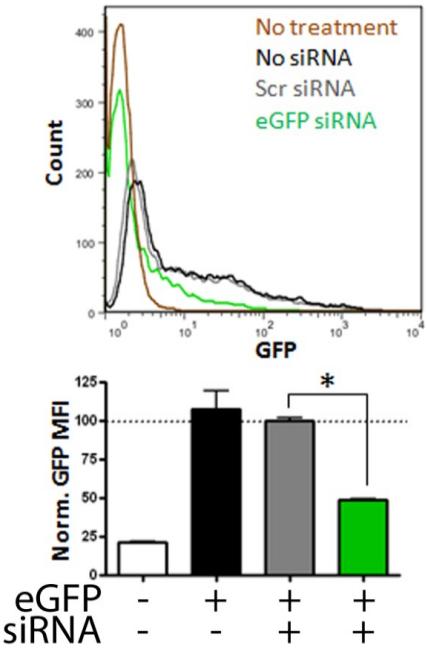

Figure 6: A549s were cotransfected with pCAGGSeGFP (eGFP) and siRNAs via nucleofection. A549s were transfected with either siRNAs targeting eGFP (G) or scrambled siRNAs with no known target sequence (S). Cells were also transfected with pCAGGSeGFP alone to control for possible effect of the scrambled siRNA on eGFP expression. The mean fluorescence intensity shown is relative to scrambled siRNA, which is normalized to 100%. Statistical significance ($P<0.05$) is indicated by a star (*).

To test for this possibility, we cotransfected A549s via nucleofection as above (Figure 6). Instead of plating each cuvette to a single well on a 6 well plate, each cuvette was divided equally between two wells. At 24 hours post nucleofection, one set of wells was mock infected while the other set was infected with rUdorn at an MOI of 5 infectious viruses per cell. The cells were harvested and fixed 24 hours post infection, and viral NS1 protein was identified in infected cells by immunofluorescence. Fluorescence indicating eGFP expression and NS1 was measured by flow cytometry. As seen in the previous nucleofection experiment, A549s cotransfected with pCAGGSeGFP and eGFP siRNA showed a significant reduction in eGFP expression in comparison

to cells cotransfected with pCAGGSeGFP and scrambled siRNAs, as indicated by a drop in overall fluorescence (Figure 7a) and a roughly twofold decrease in the mean fluorescence intensity (Figure 7b) of eGFP expressing cells. More importantly, infection was not impeded by nucleofection as is indicated by the achievement of approximately 99% of cells infected in nucleofected samples as well as the untransfected controls (Figure 7b). In addition, there was no significant difference in the reduction of eGFP expression in eGFP siRNA transfected cells that were infected compared to those mock infected in relation to their respective scrambled siRNA controls (Figure 7a and 7b). This suggests that infection does not interfere with siRNA mediated knockdown of eGFP expression in A549s, a result previously seen in the previous cell types. While the A549 cell line is more relevant for our studies, it has limitations in liposome transfection as previously presented. However, nucleofection is a suitable alternative and allows A549s to be utilized in the screening of pro-siRNAs.

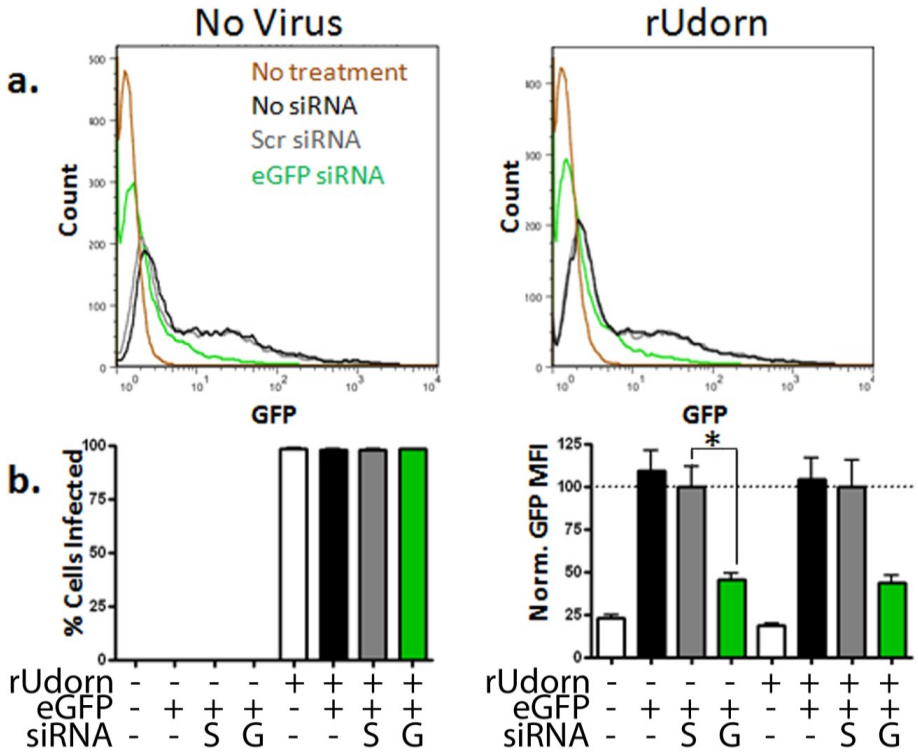

Figure 7: A549s cotransfected with pCAGGSeGFP and siRNAs via nucleofection. A549s were transfected with either siRNAs targeting eGFP (G) or scrambled siRNAs (S). Cells were also transfected with pCAGGSeGFP alone to control for possible effect of the scrambled siRNA on eGFP expression. Half the cells from each cuvette were infected with rUdorn 24 hours post transfection, while the remaining cells were mock
infected. Cells were fixed 24 hours Infected populations were identified by immunofluorescence for viral NS1 protein. Fluorescence indicating eGFP expression and infection was measured by flow cytometry. The mean fluorescence intensity shown is relative to scrambled siRNA, which is normalized to 100%. Cells cotransfected with pCAGGSeGFP and eGFP siRNAs showed a significant decrease in mean fluorescence intensity in comparison to cells cotransfected with pCAGGSeGFP and scrambled siRNAs. Statistical significance ($P<0.05$) is indicated by a star (*).

DISCUSSION

In order to develop an experimental system with which to test pro-siRNAs, we evaluated several cell types by validating the silencing activity of conventional eGFP siRNAs in each. We first explored the use of MDCK cells that stably express eGFP, demonstrating successful knockdown of eGFP expression as indicated by a 50% reduction in mean fluorescence intensity (MFI). We then transitioned to using the more relevant HEK293T cell line, showing that we could successfully cotransfect an eGFP expressing plasmid along with siRNAs simultaneously, while also demonstrating successful silencing of eGFP expression as indicated by a 60% reduction in MFI. In addition to using transient cotransfection, we also utilized 293Ts stably transfected with the VIRGS plasmid, which produces eGFP RNAs that are transcribed by the influenza polymerase. Infection resulted in eGFP expression, and we showed successful knockdown as indicated by a 50% reduction in MFI of expressing cells. Seeking a more relevant cell type for an experimental platform, we investigated the use of A549 cells, which are derived from human alveolar carcinoma, potentially a better cell type with which to evaluate therapeutics against a respiratory virus such as influenza. Using a similar cotransfection protocol to 293T cells, we were able to show effective silencing of eGFP as indicated by a 60% reduction in MFI. However, liposome transfection resulted in inhibited infection in this cell type. Using nucleofection as an alternative method of A549 cotransfection, we were able to show effective eGFP silencing as indicated by a 50% reduction in MFI without inhibition of subsequent infection. Our transfection methods using conventional siRNAs show that any of these systems could be used to test pro-siRNA designs for their ability to silence eGFP.

While knockdown of eGFP expression was successfully demonstrated in each cell type, the shifts in the MFI reductions ranged from 50% in 293T VIRGS cells to 60% in A549 cells. While this may

not be seen as a significant difference, it is likely due to variations in transfection efficiency, which could be addressed by further optimization of the amount of lipofectamine used or the plating density prior to transfection. Plating density is one of the most sensitive and most important factors of reproducible transfection (63). For the 293T VIRGS cells, 6x10^5 cells are plated per well on a six well plate roughly two days prior to transfection. Higher transfection efficiencies might result from plating a lower density, such as 3x10^5 cells per well. This did not appear to be necessary for the evaluation of the 293T VIRGS cell platform, but increased sensitivity and thus further optimization may be required if pro-siRNA silencing activity appears weak or nonexistent in this cell type.

In each of the siRNA experiments presented, eGFP expression (and in some cases infection) was measured in cell populations receiving eGFP siRNAs, scrambled siRNAs or no siRNAs at all. While a successful experiment is denoted by the characteristic decrease in mean fluorescence intensity between the eGFP siRNA and scrambled siRNA cell populations, we occasionally saw a decrease in MFI between scrambled siRNA and no siRNA populations. In some experiments, this difference was found to be statistically significant. If the scrambled siRNA does not target eGFP or any human gene sequence in cells, then why would it decrease the MFI of eGFP positive cells? There are two possible explanations. In 293Ts and A549s which are cotransfected with siRNA and an eGFP expressing plasmid, the addition of siRNA could reduce the copy number of plasmids transfected into cells, resulting in signal reduction. This can be expected when attempting to transfect two different nucleic acids into the same cells, as not all cells will receive both and those that do may receive less copies of the eGFP plasmid than cells that were transfected with the plasmid alone. However, we also have seen this effect in the VIRGS cell line, which is not cotransfected with plasmid to produce eGFP, so reduction in plasmid transfection efficiency and copy number does not always explain this difference. Another

explanation is partial silencing of eGFP due to nonspecific or 'off-target' effects. As previously explained, siRNAs can occasionally target unrelated mRNAs for degradation if even small portions of their sequences are homologous. This could interfere with eGFP expression by knocking down genes affecting its translation. These nonspecific effects can overestimate the knockdown of a gene of interest in RNAi experiments when compared to cells receiving no siRNA, which is why including a negative control siRNA should be standard procedure in RNAi studies. In our experiments, the silencing activity of eGFP siRNA was compared to that of an equivalent amount of scrambled siRNA in order to determine the true drop in eGFP expression as measured by the geometric mean fluorescence intensity (MFI). This is why we normalized our scrambled siRNA conditions to 100%, as it allowed for clearer analysis of the eGFP targeting effects of conventional and later pro-siRNAs. To measure the impact of non-specific effects on eGFP expression, we included cell populations transfected with no siRNA at all.

The second major factor in a pro-siRNA experiment is infection by influenza virus following transfection. Because pro-siRNAs should require infection to actively silence eGFP, it was crucial that we determine whether or not our transfection protocol would inhibit subsequent infection, or if infection would alter the eGFP silencing effect. To assess the compatibility of our cell type specific protocols with infection, we infected cells transfected with eGFP siRNAs and compared the reduction of eGFP expression with cells that were mock infected. In MDCK and 293T cells, transfection did not hinder infection, nor did infection significantly alter the knockdown in eGFP expression as indicated by a shift in MFI. Liposome transfection of A549s however, significantly impeded subsequent infection by influenza virus. While we did not perform a detailed analysis as to why lipofectamine2000 inhibited infection in A549s, we speculate that fusion of liposome-DNA complexes with the cell membrane altered membrane fluidity, interfering with endocytosis or fusion. Infection prior to transfection alleviated the inhibition of infection, which supported

the theory of liposome-DNA complexes being responsible. However, infection impeded the expression of eGFP from the transfected plasmids, a result that is likely explained by the ability of influenza to shut down cellular gene expression (3). Nucleofection proved to be a suitable alternative, as the transfection process did not impede subsequent infection. Infection also had no effect on the siRNA mediated reduction in eGFP expression, a result that is consistent with the other cell types investigated.

Which of these experimental systems should be used to test pro-siRNAs? To answer that, one must consider the advantages and disadvantages of each. The stably transfected MDCK cells are among the simplest of the systems presented, as they produce eGFP constitutively without the need for cotransfection. This would allow for comparison of eGFP silencing activity of pro-siRNAs with and without influenza virus infection, a critical experiment when considering the premise of infection-dependent gene silencing. The MDCK system has its drawbacks however. One such drawback is the lack of clinical relevance. While MDCK cells are the standard cell line for influenza virus propagation, they are dog kidney cells, far removed from the ideal of human respiratory epithelial cells. In addition, MDCKs are difficult to transfect, requiring 50% more lipofectamine2000 than the other cell types tested, as well as transfection in suspension in order for noticeable knockdown to occur. The extra lipofectamine results in more costly experiments, which can be an issue in a pro-siRNA experiment in which multiple designs may be screened with and without infection.

A better system for testing the pro-siRNAs might be the 293T cell line. While being a human cell line, it is a more relevant platform than the MDCK cells, and cotransfection allows for simultaneous delivery of siRNA and a plasmid continuously expressing eGFP. As shown by cotransfection of plasmid with scrambled siRNAs, the procedure itself does not result in much

change of eGFP expression. By transfecting pCAGGSeGFP along with a siRNA, 293Ts could be used to test pro-siRNA activity with and without influenza virus infection, just as the eGFP expressing MDCK cells could, with the added benefits of carrying more clinical relevance as well as being significantly easier to transfect. These cells also have the benefit of growing quickly, allowing for rapid experiment turnaround and more timely results. The main downside of using this system is that cotransfection relies on sufficient transfection efficiency of both plasmid and siRNA in order to see expression or knockdown, as both are delivered in the same liposome mix. A problem with either nucleic acid that could affect transfection efficiency may affect the transfection of both.

An alternative to the cotransfection of 293Ts is the use of the 293T VIRGS cell line to screen pro-siRNAs. These cells carry the Virus Induced Reporter Gene Segment expressing plasmid, pHH21 NP UTR Hi eGFP, which produces eGFP RNAs that mimic influenza gene segments, and are thus transcribed by the influenza polymerase. This means that eGFP is only produced in infected cells, while uninfected cells remain dark. This vastly simplifies the analysis of the potentially infection-dependent silencing activity of pro-siRNAs. In addition, stable transfection of the VIRGS plasmid gives the VIRGS cells the same advantage as the MDCK cells in that they do not require cotransfection to analyze siRNA activity, but retain the clinical relevance of human cells. The main advantage of this cell line is also its major downside however, as it can only be used to analyze siRNA activity in infected cells and is therefore unable to answer the question of whether or not any eGFP silencing activity from pro-siRNAs is infection-dependent. In addition, VIRGS cells grow far slower than their untransfected 293T counterparts due to the selective pressure required to maintain their plasmids, thus extending experiment turnaround time.

As these pro-siRNAs are being designed against influenza virus, they would be administered to cells of the human respiratory tract in a clinical setting. Therefore, an additional cell type to consider would be the A549s, which being isolated from human alveolar carcinoma are far more clinically relevant than 293Ts or MDCK cells. However, their relevance does not necessarily equate to their usefulness in a system to screen for siRNA activity. Like the unmodified 293Ts, these cells must be cotransfected with an eGFP expressing plasmid along with siRNAs. While A549s did not appear to be problematic in initial cotransfection experiments, it was intriguing that liposome transfection impeded infection in this cell type with no such similar inhibition in the other two cell types studied. Furthermore, infecting the cells with influenza virus one hour prior to transfection dramatically inhibited eGFP expression without any siRNAs at all. While such a result could be expected with what is known about influenza mediated arrest of cellular gene expression (3), it further highlights the difficulties encountered with A549 cells that never had to be considered with the other cell types.

Fortunately, nucleofection provided a means of cotransfecting A549s without inhibiting subsequent infection, potentially allowing this cell type to be used in pro-siRNA screening experiments. Nucleofection carries the advantages of being highly reproducible and transfecting cells with efficiencies that are often higher than seen with liposome based methods (62). An additional advantage of nucleofection is that transfection cuvettes can be split between wells after the procedure, allowing for the simultaneous testing of pro-siRNA mediated eGFP silencing with and without influenza infection. Being able to test infection conditions on cells from the same exact transfection procedure is perhaps the biggest advantage to using nucleofection to screen pro-siRNAs, and could not be emulated in any of the other experimental systems that were evaluated. While nucleofection is useful, it does carry drawbacks. One disadvantage of using nucleofection is the cost, with each transfection being significantly more

expensive than with liposomes. Additionally, each transfection requires roughly 10^6 cells and the cells must be subcultured 48 hours in advance such that they do not exceed 80% confluence on the day of transfection. It is also highly recommended that the procedure be completed within fifteen minutes after the cells are resuspended in nucleofector solution, or loss of viability or efficiency could result (64). These three technical drawbacks place logisitical limits on the size of an experiment, which is a considerable drawback to using this system to screen numerous pro-siRNAs and their associated controls.

To answer the question of which system would be best for testing pro-siRNAs against eGFP, one would have to consider what questions they specifically seek to answer. In order to test the general proof of principle, whether or not pro-siRNAs have any silencing activity at all, it would be best to use the 293T VIRGS cells. Limiting the investigation only to infected cells simplifies the experiment, and utilizing a stably transfected cell line ensures that any off-target effects are directly caused by the pro-siRNA in the cell and not a flaw in eGFP plasmid transfection. The 293Ts are also easier to transfect and more clinically relevant than the eGFP expressing MDCK cells. However, if the goal is to assess whether or not a particular pro-siRNA remains biologically inert in uninfected cells, one should use A549s cotransfectedvia nucleofection to compare the silencing activity of pro-siRNAs with and without virus infection.

CHAPTER 3: Evaluating Pro-siRNA Construction and Activity

INTRODUCTION

While conventional siRNAs have shown promise in antiviral therapies, it would be more ideal to use a pro-siRNA that is designed to silence genes in infected cells but not in uninfected cells. For the treatment of influenza and other cap snatching viruses, a potential design strategy would incorporate a traditional siRNA duplex with a capped 5' extension on the passenger strand. Our hypothesis is that the extension would render the molecule biologically inert, unable to silence a target gene. During infection however, viral cap snatching activity would remove the extension, leaving behind a normal duplex that could enter into the RNAi pathway. As previously stated, if such a design were to work successfully, it would restrict the pathology of off-target effects to infected cells, and would allow flexibility in the transfection dose as the active dose would be modulated by the infection itself. In order to work however, careful consideration must be made for biologically relevant details both in the design and construction of pro-siRNAs.

One of the most important details to take into account during pro-siRNA design is the preferred position and sequence specificity in the PA endonuclease activity. While PA has been shown to cleave between 10 and 13 nucleotides downstream of the cap, a finer level of specificity needs to be considered in the design of the 5' extension to ensure the most efficient activation of pro-siRNAs. *In vitro* studies by K. Datta et al. (9) have demonstrated that PA prefers to cleave after a guanine residue just before a cytosine residue, so long as the adjacent bases occur within 10-13 nucleotides downstream from the cap. In addition, the PA endonuclease showed comparatively little cleavage activity in AU rich regions. (9) With these details in mind, we designed the 5' extension of our pro-siRNAs to carry a single GC motif as a cleavage site within an AU rich sequence, the next nearest guanine residue being far removed from the range of PA activity.

The specific extension sequence we used is derived from Datta et al. as their design effectively restricted PA activity to the intended GC cleavage site, and was therefore was a verified sequence we could use to ensure cleavage site control in our pro-siRNA design (9).

Another detail to consider is the ideal position of the cleavage site relative to the 5' cap as well as the 5' start of the duplex. *In vitro* studies of polymerase complex mediated RNA cleavage show that PA most efficiently cleaves 12 nucleotides downstream of the cap if no guanine is present within the 10-13 range, but after the 13^{th} nucleotide downstream if it is a guanine followed by a cytosine (9). Therefore, our fundamental pro-siRNA design consists of a 13 nucleotide extension between the 5' cap and the PA cleavage site. Due to concerns about steric hindrance of PA activity, our initial design includes an additional 3 nucleotide spacer region between the cleavage site and the start of the duplex.

In addition to the fundamental design of the pro-siRNA, consideration must also be paid to its construction. The strategy we use to prepare the pro-siRNA duplexes begins with the enzymatic capping of chemically synthesized RNA oligos. In order for capping to take place, the oligos must carry 5' triphosphates (65). This could be a concern if the capping reaction is not 100% efficient, as 5' triphosphorylated RNA can elicit an innate interferon response via RIG-I, especially if it contains a double stranded region (66). The resulting disruption in translation would confound the measurement of eGFP knockdown from the pro-siRNAs.

Another similar concern in the construction of the pro-siRNAs is what type of cap structure to use on the 5' end. The cap structures found in nature consist of at least the N-7-methylated guanosine with no additional methylations (67). This is known as cap-0, and is primarily found in lower eukaryotes such as plants and yeast (67). Higher eukaryotes such as birds and mammals use either the cap-1 or cap-2 structures, in which the first or first two nucleotides from the N-7-

methylguanosine are 2'-O-methylated respectively (68). Producing pro-siRNAs bearing cap-0 structures would be more economical than producing them with cap-1 structures, as one would not require incorporating 2-O'-methyltransferase or additional S-adenosyl-methionine into the reaction (69, 70). However, cap-0 RNA is recognized by TLRs 7 and 8 and Mda5 in mammals and thus can trigger an innate interferon response, shutting down translation or causing apoptosis (68). Therefore, cap-0 pro-siRNAs might be problematic in that an innate interferon response could render the transfected cells refractory to infection, or result in reduced eGFP expression in infected cells. These would confound the results by limiting eGFP expression and potential pro-siRNA activation, reducing our ability to observe true silencing activity from our pro-siRNAs.

When determining whether to use cap-0 or cap-1 pro-siRNAs, it is important to consider the effects of innate interferon responses on eGFP silencing experiments. However, one must also consider which cell type is being used to conduct such experiments, as it is widely known that a number of cell lines lack complete innate interferon pathways, and therefore may not be sensitive to cap-0 RNA. As previously discussed, the ideal system with which to test pro-siRNA constructs is transfection of 293T cells stably expressing the VIRGS plasmid, because they allow for clean and simple analysis of pro-siRNA activity by expressing eGFP in a virus dependent manner. Additionally, 293Ts may be less sensitive to cap-0 RNA, as demonstrated by a group who successfully produced norovirus in 293Ts using enzymatically capped *in vitro* transcripts of norovirus genomes (71). As we would be testing for pro-siRNA activity using 293Ts, we began our experimentation using cap-0 pro-siRNAs.

After the passenger strands are capped and purified, they must be annealed to the unmodified guide strands. Besides capping, annealing is the most important step in producing functional pro-siRNAs, as the guide strand is the part of the siRNA that is ultimately loaded into RISC to

facilitate gene silencing (42). Poorly annealed duplexes will have significantly reduced silencing potency, as a siRNA mixture containing mostly single stranded RNA will have to be transfected at a higher concentration to achieve similar silencing as a mixture containing mostly duplexed siRNA (72). In addition, poor annealing could negatively impact liposome transfection efficiency due to depletion of lipofectamine by leftover single stranded RNA.

Another important decision to make in pro-siRNA development is how to test potential designs for eGFP silencing activity. While the ultimate goal would be to show that eGFP pro-siRNAs silence eGFP in infected cells but not in uninfected cells, the first step would be to establish eGFP silencing activity in infected cells. This is why 293T VIRGS cells would be used for such experiments, as they allow us to focus only on activity in infected cells, and prevent potential complications from cotransfection. Additionally, the VIRGS system synchronizes the start of eGFP expression and pro-siRNA activation, so that a silencing effect is more likely to be seen post infection.

One of the previously discussed complications from some of our pro-siRNA construction steps is the issue of non-specific effects from innate immune responses, as they can confound our results by disrupting cellular translation. While this may lead to a substantial reduction in eGFP expression, one can differentiate between true silencing and interferon mediated effects by looking at the percentage of cells infected and the MFI of viral NS1 identified by immunofluorescence. If an innate interferon response is triggered, the resulting disruption in translation should impede subsequent infection and viral protein production. While a sharp reduction in the percentage of cells infected would denote a robust innate interferon response in pro-siRNA transfected cells, a drop in the MFI of NS1 can identify more subtle responses if present. By looking at the quality of infection, it is possible to differentiate between a true

result and a false positive, which will be crucial in the interpretation of pro-siRNA experiments. With these considerations in mind, this chapter focuses on the design, construction and testing of pro-siRNAs against eGFP, with the eventual goal of evaluating the feasibility and practicality of the pro-siRNA concept as a potential therapeutic approach against influenza.

RESULTS

To test our initial pro-siRNA design for eGFP silencing activity and non-specific effects, 293T VIRGS cells were transfected with 30 pmol of conventional siRNAs or pro-siRNAs that either target eGFP or are scrambled. To assess the efficiency of our annealing procedure, the scrambled and eGFP siRNAs from Ambion were included as controls. At 26 hours post transfection, cells were infected with rUdorn virus at a multiplicity of infection (MOI) of 5 infectious viruses per cell. At 24 hours post infection, the cells were fixed and harvested. Infected cells were detected by immunofluorescence for viral NS1 protein. Fluorescence from eGFP expression and NS1 was analyzed by flow cytometry. Our first concern was whether or not the pro-siRNAs would produce non-specific silencing due to innate interferon responses to the cap-0 structure or uncapped pro-siRNAs bearing 5' triphosphates. As shown by comparison of the fluorescence profiles (Figure 8a) and mean fluorescence intensities (Figure 8b) of the different scrambled siRNAs with that of cells receiving no siRNA, the pro-siRNAs do not elicit significantly greater off-target effects than the conventional siRNAs. There was also no significant difference in the percent infection or the MFI of viral NS1 between cells receiving conventional or pro-siRNAs (Figure 8b), suggesting that infection productivity is not being altered by previous transfection of the cap-0 bearing pro-siRNAs. The above results suggest that pro-siRNAs are not causing innate interferon responses in this cell type.

While cells transfected with conventional eGFP siRNAs showed a decrease in eGFP expression compared to scrambled as indicated by eGFP MFI, cells transfected with the pro-eGFP siRNAs did not show significant eGFP silencing activity (Figure 8b). Although the annealed eGFP siRNAs produced a significant decrease compared to scrambled siRNAs, the decrease in MFI was less dramatic than seen in cells receiving the pre-annealed eGFP siRNAs from Ambion. This may

suggest that the annealing efficiency is lower than it should be. To test this, 10 pmol of pre-annealed and lab-annealed eGFP siRNA were

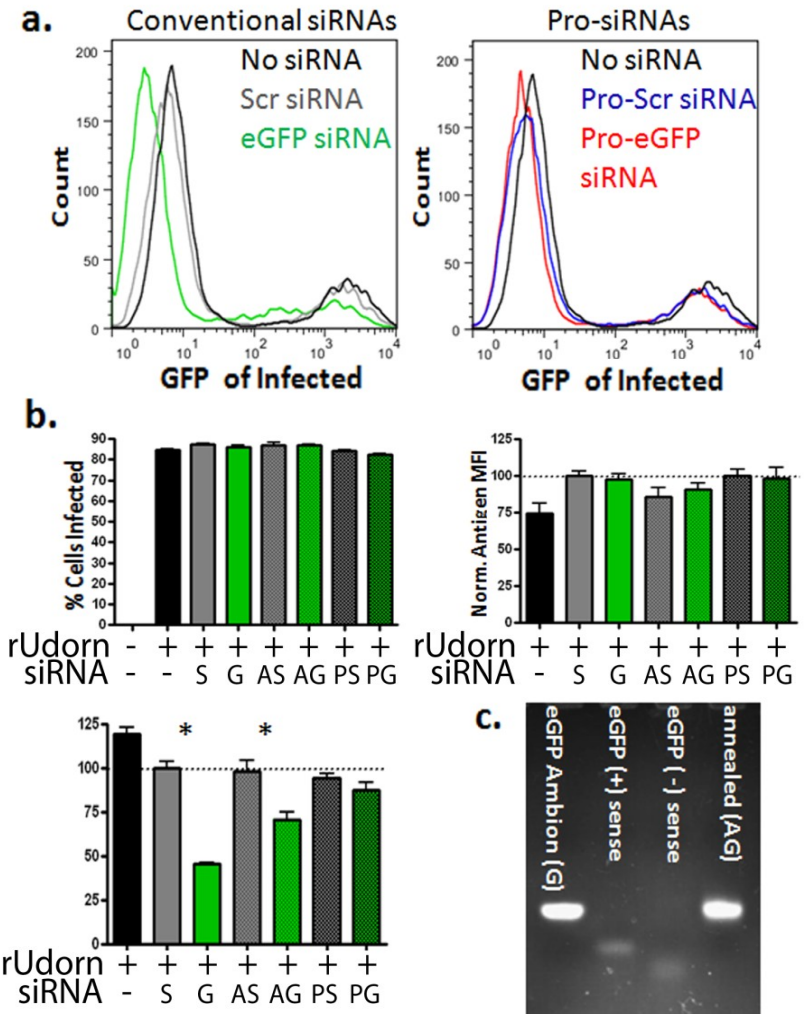

Figure 8: a,b. 293T VIRGS cells were transfected with 30 pmol of scrambled and eGFP siRNAs that were pre-annealed from Ambion (S, G), annealed in the laboratory (AS, AG) or scrambled and eGFP pro-siRNAs (PS, PG). At 25 hours post transfection, cells were infected with rUdorn virus at a multiplicity of infection (MOI) of 5 infectious virus particles per cell. Cells were fixed 24 hours post infection and infected populations were identified by immunofluorescence for viral NS1 protein. Fluorescence indicating eGFP expression and infection was measured by flow cytometry. The mean fluorescence intensity (MFI) of eGFP positive cells is shown is relative to scrambled siRNA, which is normalized to 100%. The mean fluorescence intensity of stained viral NS1 is shown relative to cells receiving no siRNA, which are normalized to 100%. **c.** 10 pmol of annealed eGFP siRNA (AG), 10 pmol of pre-annealed eGFP siRNA (G) and 20 pmol of both sense and antisense strands of the eGFP siRNA were analyzed via 12% Native PAGE, stained with ethidium bromide and visualized on a UV transilluminator. Statistical significance ($P<0.05$) is indicated by a star (*).

compared on a 12% native polyacrylamide gel with 20 pmol of guide and passenger strand oligos run in separate lanes as negative controls. After completion of electrophoresis, the RNA bands were visualized using ethidium bromide. As ethidium bromide primarily stains nucleic acids as an intercalating agent, duplexes show up brightly while single strands are faint if present at all. While the lab-annealed duplex band appears to be somewhat fainter than the pre-annealed band, its brightness and definition suggest that the annealing reaction should be efficient enough for our purposes.

While troubleshooting needed to be done to investigate the lack of eGFP silencing activity in our pro-siRNAs, improvement of eGFP silencing via conventional siRNAs might allow more sensitive visualization of pro-siRNA activity if it exists. Therefore, we first sought to improve the knockdown seen with the pre-annealed and lab-annealed siRNAs in the VIRGS cell system. Previous experiments suggested that low confluence at the time of transfection resulted in greater than expected eGFP silencing activity from transfected siRNAs. (Data not shown) This was thought to be due to high transfection efficiency at that density. To investigate the possible boost in transfection efficiency that may occur from plating at a lower density, 293T VIRGS cells were plated at densities of both 6x10^5 and 3x10^5 cells per well on six well plates and were transfected with pre-annealed eGFP or scrambled siRNAs. At 25 hours post transfection, the cells were infected with an MOI of 5 infectious viruses per cell. Cells were fixed and harvested at 24 hours post infection and infected cells identified by immunofluorescence for viral NS1 protein. Fluorescence from eGFP expression and viral NS1 was analyzed via flow cytometry. While the eGFP MFI reduction observed in both conditions was less dramatic than expected, there was no significant increase in eGFP silencing in 293T VIRGS cells plated at 3x10^5 cells per well in comparison to cells plated at 6x10^5 per well. This suggests that plating at 3x10^5 cells

per well does not improve knockdown results. Therefore, subsequent experiments would be performed using the original plating density of 6x10^5 cells per well.

A second means of optimizing siRNA transfection is to determine the optimal amount of siRNA to transfect into cells. Up to this point, each cell population had been transfected with 30 pmol of siRNA. While previous experiments using cotransfection did not show any increase in eGFP

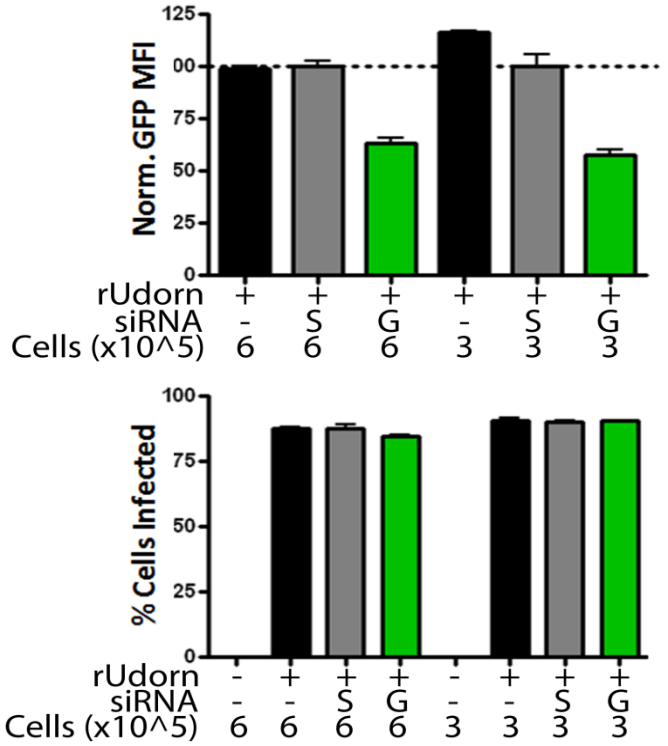

Figure 9: An analysis of the effect of transfection on cell density by comparing multiple replicates. To differentiate between experiments, the date of each is provided below the respective columns. In each experiment, 293T VIRGS cells were transfected with 30 pmol of either siRNA targeting eGFP (G) or scrambled siRNA (S). At 25 hours post transfection, the cells were infected with rUdorn virus at an MOI of 5 infectious viruses per cell. At 24 hours post infection, the cells were fixed and stained for viral NS1 protein (not shown) and analyzed by flow cytometry. The mean fluorescence intensities shown are relative to the scrambled siRNA conditions, which are normalized to 100%.

silencing with increasing pre-annealed siRNA load (data not shown), increasing the amount of lab-annealed siRNAs might compensate for any reduction in potency compared to the pre-annealed siRNAs. To investigate the potential increase in knockdown from adjusting the siRNA concentration, 293T VIRGS cells were transfected with 30, 60 or 90 pmol of scrambled and eGFP siRNAs that were either pre-annealed or annealed in the laboratory. At 25 hours post transfection, the cells were infected with rUdorn virus at a multiplicity of infection of 5 infectious viruses per cell. At 25 hours post infection, the cells were harvested and fixed, and infected cells were identified by immunofluorescence for viral NS1 protein. Fluorescence from

eGFP expression and NS1 was measured by flow cytometry. As had been found previously, increasing the amount of pre-annealed eGFP siRNA delivered beyond 30 pmol had no effect on the reduction of the eGFP MFI (Figure 10). Contrary to our expectations however, increasing the amount of lab-annealed eGFP siRNA did not show any effect on eGFP MFI reduction either (Figure 10).

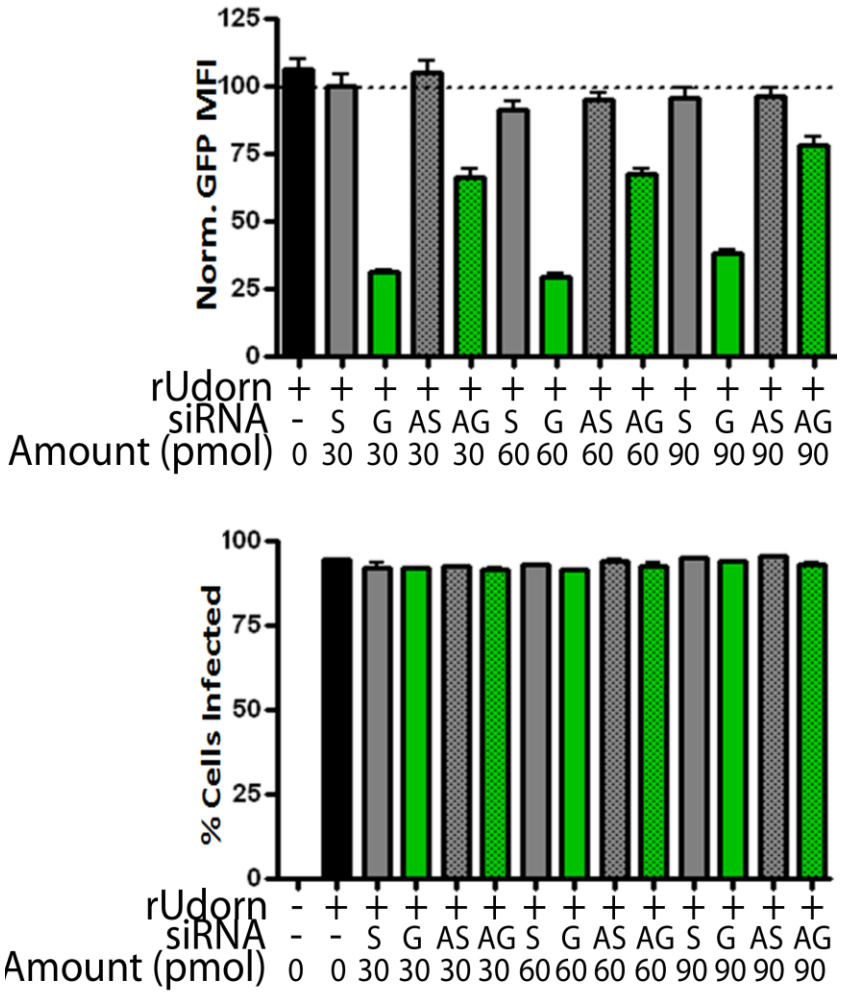

Figure 10: 3x10^5 293T VIRGS cells were transfected with scrambled and eGFP siRNAs that were pre annealed (S, G), or annealed experimentally (AS, AG) at a final concentration of 30nM (standard), 60nM or 90nM. At 24 hours post infection, cells were infected with rUdorn virus at an MOI of 5 infectious viruses per cell. At 24 hours post infection, cells were fixed and stained for viral NS1 protein, and fluorescence measured via flow cytometry. The mean fluorescence intensities shown are relative to the 30nM pre-annealed scrambled (S) condition, which was normalized to 100%.

While the previous experiment focused on adjusting the amount of siRNA transfected, the amount of lipofectamine used in each transfection was kept constant at 4 µL per well. To account for the possibility that lipofectamine limited the transfection of higher amounts of

siRNA, 293T VIRGS cells were transfected with 90 pmol of scrambled and eGFP siRNA that was either pre-annealed or annealed in the laboratory, using 2, 4, 8 or 12 µL of lipofectamine2000 per well. At 25 hours post tranfection, cells were infected with rUdorn virus at an MOI of 5 infectious viruses per cell. Cells were harvested and fixed 24 hours post transfection and infected cells were indentified via immunofluorescence for viral NS1 protein. Fluorescence from eGFP expression and viral NS1 was measured by flow cytometry. Contrary to our hypothesis, increasing the amount of lipofectamine used in siRNA transfections did not significantly alter the knockdown of eGFP expression with lab-annealed siRNAs, while increasing lipofectamine beyond 4 µL per well appeared to decrease knockdown activity in the pre-annealed siRNAs. (Figure 11) These experiments show that our original transfection parameters of plating 6x10^5 293T VIRGS cells per well, and delivering 30 pmol of siRNA using 4 µL of lipofectamine should be sufficient to observe optimal eGFP silencing activity of lab-annealed conventional and pro-siRNAs. The lack of potency of lab-annealed eGFP siRNAs is still a concern, however we can use their existing eGFP silencing effects to investigate potential improvements to the construction and application of pro-siRNAs.

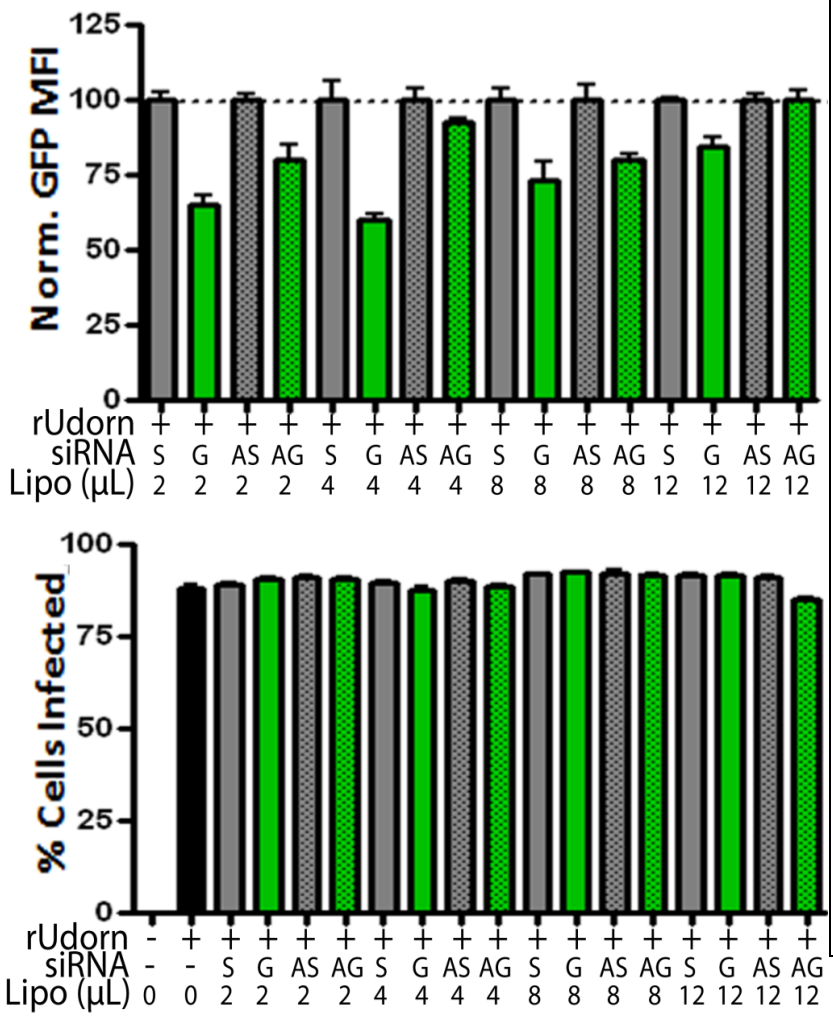

Figure 11: 293T VIRGS cells were transfected with scrambled and eGFP siRNAs that were pre annealed (S, G), or annealed experimentally (AS, AG) at a final concentration of 90nM using 2, 4, 8 or 12 µL of lipofectamine per well. At 24 hours post infection, cells were infected with rUdorn virus at an MOI of 5 infectious viruses per cell. At 24 hours post infection, cells were fixed and stained for viral NS1 protein, and fluorescence measured via flow cytometry. The MFI of each eGFP siRNA condition is shown relative to its scrambled condition, which is normalized to 100%.

After attempting to overcome the reduced potency of lab-annealed conventional siRNAs, we sought to investigate the complete lack of eGFP silencing activity from our initial pro-siRNA design. As previously mentioned, one of the most important design variables is the position of the PA cleavage site relative to the 5' cap, which can have a significant effect on cleavage efficiency and thus conversion of innert pro-siRNAs into active siRNA duplexes. Therefore, we used a series of previously designed pro-siRNAs with 5' extension lengths ranging from 11-14 nucleotides between the cap and the cleavage site. (Figure 12)

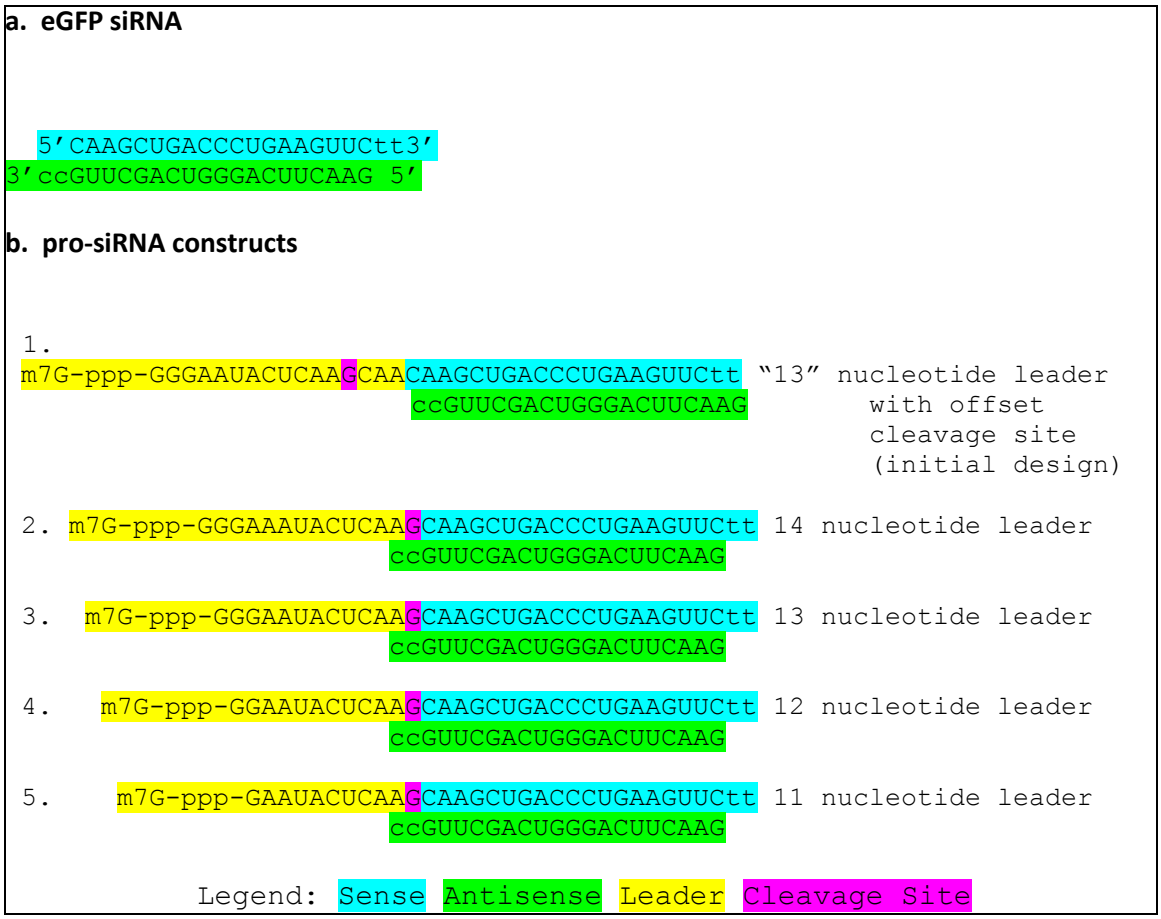

Figure 12: a. The eGFP siRNA of functional length used during screening assay validation. **b.** The pro-siRNA constructs to be tested incorporate the sequence of the eGFP siRNA (a) with the 5' addition of a capped leader sequence (yellow) ranging from 11-14 nucleotides (1-5). The cleavage site of the first construct is offset from the duplex to relieve possible steric hinderance. The spacer region was omitted from the four subsequent designs (2-5) All initial pro-siRNA experiments were conducted with a scrambled and eGFP targeting variant of design #1 shown above.

The spacer region between the cleavage site and the duplex in our initial design was omitted from these designs as they did not account for the potential steric hindrance of PA activity.

To evaluate the role of extension length in our design, 293T VIRGS cells were transfected with 90 pmol of conventional scrambled or eGFP siRNAs that were pre-annealed or annealed in the laboratory, 90 pmol of eGFP pro-siRNAs with distances between cap and cleavage site ranging from 11 to 14 nucleotides, and our initial eGFP and scrambled pro-siRNAs utilizing a 13

nucleotide extension plus the 3 nucleotide spacer region. At 25 hours post transfection, cells were infected with rUdorn virus with an MOI of 5 infectious viruses per cell. At 24 hours post infection, the cells were harvested and fixed, and infected cells were identified via immunofluorescence for viral NS1 protein. Fluorescence from eGFP expression and NS1 immunofluorescence was analyzed by flow cytometry. The results shown are a pool of two replicate experiments, each done in triplicate. As seen previously, there was no eGFP silencing activity from our initial pro-siRNA design ("P1" Figure 13) as indicated by the lack of eGFP MFI reduction in comparison to the scrambled pro-siRNA. However, the designs with 14 (P2), 13 (P3) and 12 (P4) nucleotide extensions between the cap and the cleavage site at the start of the duplex produced a modest but significant reduction in eGFP MFI in comparison to the scrambled control (Figure 13). There was no significant difference in performance between the 14, 13 and 12 nucleotide design variants, suggesting that in theory, PA may cleave their extensions with similar efficiency. However, the 11 nucleotide extension variant (P5) did not show significant eGFP silencing activity, suggesting that 12-14 nucleotides is the ideal length between the cap and the cleavage site in our pro-siRNAs. The eGFP silencing activity observed in these constructs (P2-4) and the lack of silencing activity in our initial design (P1) suggest that the 3 nucleotide spacer region may interfere with pro-siRNA activity. While the results from P2-4 are promising, it is important to consider the role of innate immune responses on percieved pro-siRNA mediated silencing. To assess whether or not innate interferon responses were contributing to eGFP knockdown, the MFI of NS1 immunofluorescence were compared between the pro-siRNA conditions. As found with our initial design, none of our pro-siRNAs produced a significant reduction in NS1 MFI compared to each other, or cells receiving conventional siRNAs or no siRNA at all (Figure 13). This suggested that the modest reduction in eGFP expression was due to true pro-siRNA activity. While further work will need to be done to optimize eGFP silencing

with pro-siRNAs as well as to test pro-siRNAs in non-infected cells, the fact that we saw activity in infected cells suggests that the pro-siRNA concept might be feasible.

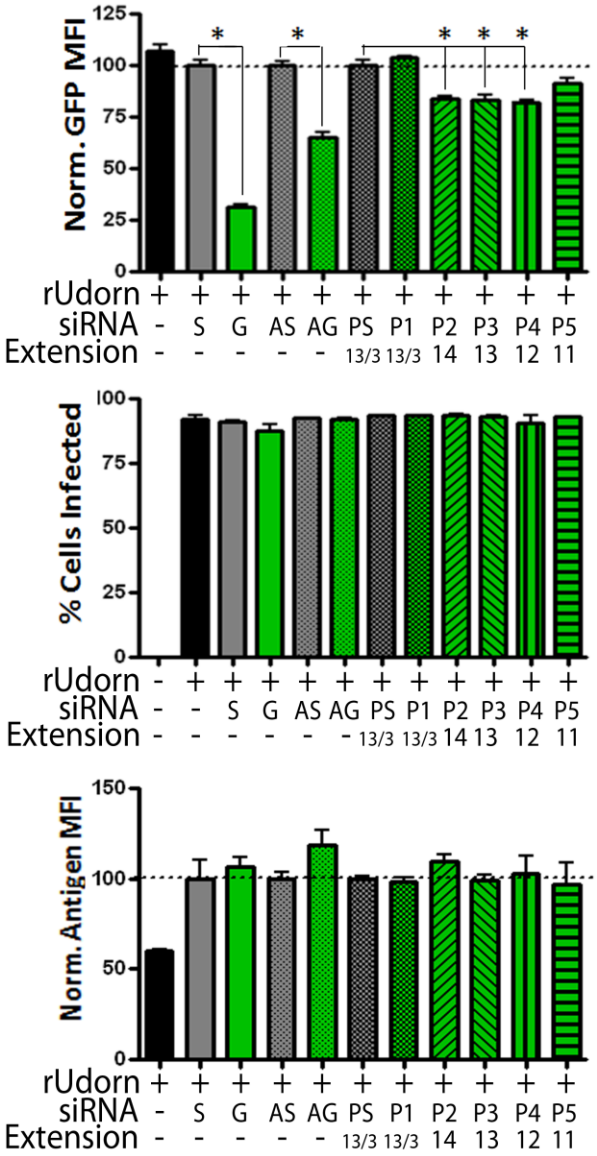

Figure 13: 293T VIRGS cells were transfected with 90 pmol of conventional scrambled and eGFP siRNAs that were pre-annealed (S, G) annealed in the laboratory (AS, AG), scrambled pro-siRNAs (PS) or eGFP pro-siRNAs (P1-P5) with varying capped 5' extension lengths. The numbering scheme corresponds to the design numbers shown in Figure 12, with P1 containing 13 nucleotides between the cap and cleavage site, P2 carrying a 14 nucleotide extension, P3 carrying a 12 nucleotide extension, P4 carrying an 11 nucleotide extension and P5 carrying the same 5' extension as P1 but with an additional 3 nucleotide spacer between the cleavage site and the duplex. At 24 hours post transfection, cells were infected with rUdorn virus at an MOI of 5 infectious viruses per cell. At 24 hours post infection, cells were fixed and stained for viral NS1 protein, and fluorescence measured via flow cytometry. The eGFP and viral antigen MFIs of each eGFP siRNA condition is shown relative to its scrambled condition, which is normalized to 100%. The No siRNA condition is shown relative to the pre-annealed scrambled siRNA (S) condition. In this figure, a star (*) indicates statistical significance of $P<0.001$.

DISCUSSION

The previous chapter describes four different experimental systems that were validated using conventional siRNAs. These were liposome mediated transfection of siRNAs into MDCK pCAGGSeGFP or 293T VIRGS cells, liposome cotransfection of siRNAs and pCAGGSeGFP plasmid into 293T cells, and nucleofection of siRNAs and pCAGGSeGFP into A549 cells. Of these options, we chose to perform the initial pro-siRNA experiments using the 293T VIRGS cells due to their infection dependent expression of eGFP. Use of a stably transfected cell line eliminates the need for cotransfection, simplifying the assay and reducing alterations in transfection efficiency that could produce confounding results. Use of the VIRGS system has additional advantages, as infection dependent expression of eGFP allows for cleaner and clearer analysis of eGFP silencing activity of pro-siRNAs. As pro-siRNAs should require virus to be active, we were initially interested in analyzing the effects of pro-siRNAs in infected cells, which are the only cells producing eGFP when using the VIRGS system. One could argue that we could use pCAGGSeGFP to continuously express eGFP while gating out cells not expressing viral NS1 protein. While this would allow for analysis of eGFP expression in infected cells specifically, one would be less likely to observe pro-siRNA mediated knockdown of eGFP expression, as eGFP production would begin 24 hours prior to pro-siRNA activation via infection. Though our MDCK pCAGGSeGFP experiments suggest that it may be possible to observe such knockdown in cells continuously expressing eGFP, it would be difficult to compare pro-siRNA activity to that of conventional siRNAs, which would silence eGFP starting at transfection as opposed to infection 24 hours later.

For the initial evaluation of pro-siRNA activity, pro-siRNAs and conventional siRNAs were transfected into 293T VIRGS cells. The conventional siRNAs were either pre-annealed or annealed in the laboratory using the previously outlined protocol. Using siRNAs annealed with

the same procedure as the pro-siRNAs controlled for annealing efficiency and duplex quality, which were evaluated via comparison to the activity of pre-annealed siRNAs. As the pro-siRNAs carried monomethylated caps (cap-0) or 5' triphosphates, both of which could trigger innate interferon responses (68), it was crucial to monitor for possible non-specific effects that could result in cytotoxicity or otherwise obscure true eGFP silencing. For that reason, we used a scrambled pro-siRNA with a sequence identical to that of the scrambled conventional siRNAs. Through this combination of controls, we were able to show that our initial pro-siRNA design did not silence eGFP as was hypothesized. In that same experiment, the lab-annealed conventional eGFP siRNA only produced half the eGFP MFI reduction of the pre-annealed eGFP siRNA, suggesting that something was wrong with the annealing step. However, analysis via native PAGE suggested that the annealing reaction was successful.

While we could not directly explain the significantly reduced potency of the lab-annealed siRNAs compared to the pre-annealed siRNAs, we thought that further optimization of transfection conditions could compensate for the weaker eGFP silencing effects. This way, we may be able to see pro-siRNA activity if it was present but otherwise undetectable using our standard transfection parameters. The first variable we tested was cell density, due to a previous observation that suggested low density may increase the eGFP knockdown effect by increasing the available siRNA per cell. We therefore transfected pre-annealed eGFP siRNAs into 293T VIRGS cells that had been plated at either 6x10^5 or 3x10^5 cells per well. This demonstrated that cell density below 6x10^5 cells per well did not have a significant effect on eGFP silencing activity. While we only reduced the plating density by half, significantly further reduction would result in too few cells for sufficient analysis via flow cytometry after the typical amount of cell-death from transfection and infection. The next variable we investigated was the amount of siRNA transfected. While previous experiments showed no increase of eGFP silencing with more

than 30 pmol of pre-annealed eGFP siRNAs, we thought that the reduced potency of our lab-annealed siRNAs could be overcome by transfecting a higher dose. However, increasing the dose as high as 90 pmol did not produce any greater eGFP silencing effect. One problem with this experiment is that we did not increase the amount of lipofectamine used in each condition. This amount had been previously used for cotransfection of 1 μg of plasmid DNA in addition to the 30 pmol of siRNA. Therefore, we thought that it was in excess, and therefore did not need to be increased with siRNA dose. As our results did not show any increase in eGFP silencing with increasing dose of lab-annealed siRNAs, it was thought that the amount of lipofectamine might be a limiting factor for the higher doses of siRNA. Therefore, we next tried transfecting 90 pmol while varying the amount of lipofectamine from as low as 2 μL to as high as 12 μL per well. The results show no significant increase in lab-annealed siRNA activity with increasing lipofectamine dose, while also showing decreasing eGFP silencing activity of pre-annealed siRNA with increasing dose. This suggests that transfection efficiency may actually be reduced by increasing the amount of lipofectamine used, contrary to our hypothesis. As the original amount of lipofectamine used is 4 μL to transfect 30 pmol of siRNA, it is interesting to note that 12 μL to transfect 90 pmol of siRNA gives such a poor result, as they use the same ratio of lipofectamine to siRNA. A possible explanation is that the overall concentration of lipofectamine was too high in the transfection mixture. We did notice aggregate debris in wells transfected with 12 μL of lipofectamine while no such debris was observed in wells receiving lower amounts, suggesting aggregation as a plausible mechanism for reduced transfection efficiency at higher lipofectamine doses. This might be overcome by increasing the volume of the transfection mixture so that a higher dose of lipofectamine and siRNA complexes could be delivered at a similar concentration to the lower doses previously used. However, not increasing the concentration may counteract the increase in the amount of siRNA and lipofectamine applied.

One of the main limitations going forward will be the potency of our lab-annealed siRNAs. While the pre-annealed siRNAs usually produce a 50-60% reduction in eGFP MFI, the lab-annealed siRNAs produce a 30% reduction in eGFP MFI at best. While one might suspect that our annealing protocol is not effective, similar protocols have been successfully used by other authors, (73) and successful annealing was confirmed in our laboratory via native PAGE. One caveat to our analysis however, is that we stain using ethidium bromide. As an intercalating agent, it readily stains duplexes while showing less sensitivity in detecting ssRNA (74). If an annealing reaction is only 75% efficient, it may not detect the remaining ssRNA. A better dye to use would be SYBR Gold, which can detect both single strand and duplex RNA with high efficiency (75) and may therefore provide a better means of checking the annealing efficiency.

In theory, poor annealing of siRNA duplexes would result in reduced potency per picomole of transfected siRNA due to the reduced amount of active siRNA duplexes. In the same vein, partial degradation of the strands would also reduce potency. If reduced siRNA potency were attributable to these causes however, one would expect a twofold or threefold dose increase to have a noticeable effect on eGFP silencing activity, as the dose of properly annealed duplexes would be higher. Interestingly, our results showed no increased eGFP silencing with increased siRNA dose. Another possibility would be that single stranded material from poorly annealed or degraded siRNA preparations would consume lipofectamine, reducing the transfection efficiency of the remaining siRNA duplexes. However, this should be overcome by increasing the concentration of lipofectamine used in the transfection mixture, while results show no increase in eGFP silencing activity when our lab-annealed siRNAs are transfected with increasing amounts of lipofectamine2000. Therefore, there may be a systematic problem with our lab-annealed eGFP siRNAs that is not dependent on transfection dose. This could be investigated by

fluorescently labeling either the guide or passenger strands to visualize siRNA localization in cells via fluorescence microscopy.

Though we could not overcome the lack of potency of our lab-annealed siRNAs, it should be noted that they still produced statistically significant reductions in eGFP expression, as seen by decreasing MFIs of eGFP positive cells. While their reduced potency was not ideal, the lab-annealed eGFP siRNAs still provided a useful benchmark for evaluating pro-siRNA silencing activity. The lab-annealed eGFP siRNAs never produced greater than a 40% reduction in eGFP MFI, while the initial pro-siRNA design did not show any activity at all. As previously discussed, this design utilizes a 13 nucleotide leader sequence between the 5' cap and the PA cleavage site, as well as a 3 nucleotide spacer region between the cleavage site and the first nucleotide of the duplex. The space between the cap and the cleavage site is supported by the work of Datta et al., which showed that the PA endonuclease most efficiently cleaves RNA after a guanine as the 13^{th} nucleotide downstream from the 5' cap (9). The spacer region between the cleavage site and the duplex was included to alleviate potential steric hindrance of PA endonuclease activity. As this design did not show any eGFP silencing activity during our initial experiments, we decided to test a series of other pro-siRNA designs, with the PA cleavage site positioned from 11 to 14 nucleotides downstream of the 5' cap. These were originally designed without concern for steric hindrance of PA, and thus their cleavage sites are positioned at the start of the duplex region. To ensure that any activity would be seen if present, 293T VIRGS cells were transfected with 90 pmol each of the four new pro-siRNA designs as well as the initial design and scrambled control. Although it was previously found that transfecting more than 30 pmol of conventional eGFP siRNA did not result in increased knockdown, 90 pmol of pro-siRNAs were used so that an effect could be seen if inefficient but successful PA mediated pro-siRNA activation was occurring. Interestingly, the designs with 12, 13 and 14 nucleotide extension lengths showed

between 15 and 20% reduction in eGFP expression while the 11 nucleotide extension design and our initial design did not show significant silencing activity. While 15%-20% silencing of overall eGFP expression may not appear to be significant, it is half the activity shown by our lab annealed siRNAs. As the three 'active' designs did not show any differences in silencing between one another, these results suggest that the ideal range of PA mediated cleavage of a pro-siRNA is between 12-14 nucleotides downstream of the cap. This generally concurs with the findings of Datta et al. that PA ideally cleaves after the 13th nucleotide if it is a guanine, but will cleave any guanine within a range of roughly 10-13 nucleotides (9). However, that does not explain why the design with the 11 nucleotide extension lacked the same eGFP silencing activity as the guanine in its cleavage site is within the 10-13 nucleotide range. One possible explanation for the discrepancy is that the presence of the duplex inhibits efficient PA endonuclease activity below 12 nucleotides downstream, while mRNAs might not show similar properties unless they have highly stable secondary structures near their 5' ends. While eGFP silencing activity was hypothesized in the 13 nucleotide extension designs, it was surprising that the design with no spacer between the cleavage site and duplex produced a modest knockdown while the design with the 3 nucleotide spacer region did not reduce eGFP expression. This result appears to run contrary to our hypothesis that the design with less potential steric hindrance of PA would show greater eGFP silencing activity. In fact, it appears that the duplex structure and 3' overhang of the guide strand did not cause as much steric hindrance of PA as we previously thought it would. However, the roughly twofold decrease in eGFP silencing activity compared to lab-annealed conventional siRNAs could suggest that some steric hindrance is occurring; reducing the efficiency of PA mediated cleavage. If that is the case, why would the initial design with the cleavage site spaced away from the duplex fail to show eGFP silencing activity, with steric hindrance of PA reduced? While PA could be cleaving the initial design efficiently, the

remaining three nucleotide extension on the passenger strand of the duplex could be interfering with RISC loading, preventing eGFP silencing.

Our most recent data suggest that certain pro-siRNA designs can reduce eGFP expression, which may be due to activation of pro-siRNAs against eGFP via the influenza PA endonuclease. This implies the potential feasibility of the capped pro-siRNA concept as a possible therapeutic approach, however further investigation is needed before a final conclusion can be made. For example, we have been assuming that the pro-siRNA activity seen in infected cells is due to PA endonuclease mediated cleavage of the capped extensions based on the observation that certain designs (P2-4) have shown eGFP silencing activity while others (P1, P5) have not. While our fluorescence based assay allows for the evaluation of pro-siRNA activity, it cannot directly attribute said activity to the PA mediated cleavage that we predict. To be sure that pro-siRNAs are being activated by PA endonuclease activity, we would use the *in vitro* endonuclease assays as described in Datta et al, {{13 Datta, K. 2013}} in which pro-siRNAs with P^{32} labeled caps would be incubated at room temperature with recombinant RNPs containing the influenza polymerase complex, and reaction produces resolved and visualized on a 20% polyacrylamide gel containing 7 M urea. Such experiments would also allow us to investigate pro-siRNA cleavage efficiency, a potential cause for reduced eGFP silencing activity compared to conventional eGFP siRNAs. Suboptimal cleavage efficiency *in vitro* would suggest that further design optimization is needed. If PA mediated cleavage is efficient *in vitro*, the reduced potency of pro-siRNAs could be due to other causes including insufficient access to PA, which is confined to the nucleus. These will be discussed later.

In addition to evaluating PA mediated cleavage, further experimentation would be required before one could make a final conclusion regarding the feasibility of the pro-siRNA concept.

One such study would aim to see whether or not pro-siRNAs truly require infection to be active. Silencing activity in uninfected cells would imply that the pro-siRNA remains active even with the capped extension hypothesized to render it inert. If the extension failed to keep pro-siRNAs out of the RNAi pathway, our experiments should have been showing eGFP silencing activity in each of our pro-siRNA constructs comparable to the lab annealed eGFP siRNAs. However, experiments with our nonfunctioning initial design and 11 nucleotide extension design suggest that the capped extension does prevent entry of the duplex into the RNAi pathway, as it did not show any eGFP silencing activity. Three of the other pro-siRNA designs did show activity, but the 15-20% knockdown does not approach the 40% knockdown seen with the lab-annealed conventional siRNAs in that same experiment.

While we can reason that eGFP silencing by pro-sIRNAs is due to their activation by virus, one major caveat in our experiments is that we used the VIRGS system, which only produces eGFP in infected cells. This prevents us from looking at pro-siRNA activity in uninfected cells, which is necessary to truly verify infection-dependent eGFP silencing. Therefore our next experiment should be to cotransfect 90 pmol of the active pro-siRNA designs with pCAGGSeGFP into 293T cells to evaluate eGFP silencing activity without infection. In the previous chapter, it was explained that nucleofection of A549s would be the best means for conducting this experiment, as one can directly compare knockdown of eGFP expression between infected and uninfected cells from the same transfection. While that may still be true, a simpler approach would be to use 293T cells, as they have been shown not to respond to cap-0 RNA. Additionally, it is not necessary to make a direct comparison when the goal is to see whether or not pro-siRNAs silence eGFP at all in uninfected cells.

If the infection-dependence of pro-siRNA activity is confirmed, our previous data suggests that further optimization of construction is required. Our goal would be to improve the observed eGFP knockdown of our pro-siRNAs to be comparable to the silencing seen in lab-annealed conventional siRNAs. To do so, there are a number of variables to consider. The first of which is the cap structure used on our pro-siRNAs. Our design utilizes the cap-0 structure, which is simpler to produce enzymatically. Our initial concern with cap-0 pro-siRNAs was that they could elicit innate interferon responses to nonspecifically affect eGFP expression as well as inhibit the process of infection. However, Yunus et al. introduced cap-0 RNA into 293T cells with no significant interferon responses detected (71). While we did not test for the activation of innate interferon responses in our 293Ts transfected with pro-siRNAs, we did not find any nonspecific decrease of eGFP expression or significant reduction in viral NS1 production. This suggests that innate interferon responses were not occurring, as they would otherwise disrupt translation. The cap-0 structure may be suitable for pro-siRNAs transfected into 293T cells, but is it sufficient for efficient PA mediated cleavage? One could argue that the 2'-O-methylated cap-1 structure would be required for efficient endonuclease activity, as such a structure is used in birds and mammals in which the virus evolved (76). While cap-1 RNA is required for productive viral transcription, the influenza polymerase complex cleaves both cap-1 and cap-0 RNA efficiently (77, 78). However, it has been found that influenza PB2 may preferentially bind the cap-1 structure (77). This may be a concern in the cellular environment, where cap-0 pro-siRNAs would be competing with cap-1 pre-mRNAs for influenza polymerase complexes. Using the cap-1 structure therefore may not be required to see knockdown, but should be considered as a possible improvement to our pro-siRNA design. While it is important to consider the optimization of cap structure, the efficiency of the capping reaction should also be evaluated. Uncapped pro-siRNAs will not be able to be activated by the influenza PA endonuclease activity,

and the 5' triphosphates can elicit innate interferon responses (68, 76). While the manufacturer of our enzymatic capping kit claim that the expected capping efficiency can approach 100%, such variables as starting RNA concentration and reaction time may need to be optimized. Evaluation of capping efficiency can be performed by using α-P^{32}-GTP, allowing for detection and quantification of capped sense strand oligos after purification.

Many of the problems that we have been considering with the development and testing of pro-siRNAs against eGFP have to do with assessing their construction. Chemically synthesized passenger (sense) strands bearing 5' triphosphates are enzymatically capped *in vitro*, purified to remove unincorporated GTP and other reaction components, then combined with unmodified guide strands and annealed. Each of these steps has opportunities for reduced pro-siRNA potency, such as RNA degradation, inefficient capping, suboptimal annealing and inappropriate selection of cap structure. Therefore, each of these steps may require further experimentation and optimization before a truly effective pro-siRNA can be produced.

An appealing alternative would be to test the pro-siRNA designs using plasmid vectors expressing short hairpin (sh)RNAs. In theory, one should be able to produce a "pro-shRNA" by encoding a transcript that will form a 21-24 nt hairpin with 10-13 nucleotides on the 5' end that remain unhybridized. The spontaneous formation of the hairpin alleviates concerns about annealing efficiency. If synthesized via RNA Polymerase II, capping occurs as part of the typical transcription process (79), eliminating concerns of cap structure and capping efficiency. While using shRNA vectors could relieve us of the complexities of producing synthetic pro-siRNA duplexes *in vitro*, the pro-shRNA could require significant troubleshooting of its own. The major obstruction to the feasibility of a pro-shRNA is in the shRNA pathway. Once the vector plasmid has been transfected into the nucleus, it expresses pre-shRNAs, which are then converted into

shRNAs by Drosha, an RNAse III type enzyme that removes the excess sequences leaving a clean hairpin with a 3' overhang of roughly 2 nucleotides (80). This would mean that any 5' cap and extension sequence as part of the "pro-shRNA" design would be cleaved off, defeating any potential for infection dependent activation via the influenza PA endonuclease activity. A potential solution to this limitation however, has been investigated by Xie et al. in the characterization of a micro (mi)RNA called miR320. Human and mouse pre-miR320 are transcribed via RNA polymerase II, and carry 5' caps (79). The synthesis pathway does not involve end cleavage by Drosha as in other miRNA pathways, and the resulting molecule exported to the cytosol is similar to a short hairpin RNA that retained its 5' cap (79). While further work may be necessary to fully understand the workings of this novel miRNA pathway, development of vectors expressing similar molecules could lead to a pro-siRNA type molecule that could be reliably synthesized *in vivo*.

Whether a pro-siRNA design is successfully constructed *in vitro* using an optimized protocol, or successfully expressed in transfected cells, the overall effectiveness of the concept remains to be seen. Our current results suggest that the pro-siRNA concept is feasible, but that further optimization is needed for it to be effective as a potential treatment strategy. While we are hopeful that optimization of design and construction will result in a pro-siRNA that silences eGFP in infected cells with similar potency to conventional siRNAs, there are possible biological barriers to success with the overall concept. One reason why we may see less efficient eGFP knockdown via pro-siRNAs is if they are not adequately retained in the nucleus at the time of infection. Liposome transfection has been shown to efficiently deliver siRNAs to both the nucleus and cytosol of the cell (63). Over time however, siRNAs are excluded from the nucleus in an exportin-5 dependent manner (81). This is important, as in our experiments, the cells were infected 24 hours after transfection. If pro-siRNAs are also shuttled out of the nucleus via

exportin-5 like traditional duplexes, they would be depleted from the nucleus by the time infection took place. As influenza cap snatching occurs primarily in the nucleus, the exported pro-siRNA duplexes would remain inactive, reducing the overall potency of the dose. One could test for this problem by transfecting cells with fluorescently labeled pro-siRNAs and track their location in the cell at 6 and 24 hours using immunofluorescence microscopy. A solution to the problem could be to infect sooner after or prior to pro-siRNA transfection. As pro-siRNA based therapeutics would be delivered after infection has occurred, nuclear exclusion may not equate to unsuccessful treatment, though it could lead to less efficient activation of the siRNA and require a higher dose. However, nuclear exclusion would prevent the prophylactic use of exogenously delivered pro-siRNAs, as the nuclear concentration may be too low by the time infection takes place. Prophylaxis could instead be achieved by expressing pro-siRNA type molecules from plasmid vectors.

While attempting to work out the approach of using a capped pro-siRNA to limit gene silencing activity to infected cells, one could consider other approaches as means of achieving an infection-dependent RNAi effect. The simplest of these would be to use a plasmid that produces shRNAs off of a viral promoter. Such a strategy would be highly effective as shRNA production is tied to the expression of other regulated viral genes. As an example, adenoviruses could be treated with infection-dependent RNAi by administering plasmids expressing shRNA via an E1A driven promoter, as E1A is involved in the expression of adenoviral early genes (3). Another example would be HIV, in which transcription is regulated by the TAT protein (1). In fact, a TAT dependent shRNA vector has been developed by Unwalla et al. that targets the HIV rev protein, resulting in successful inhibition of HIV replication in cultured T-lymphocytes (82).

It is tempting to consider such an approach for infection-dependent RNAi against influenza. However, influenza promoters are recognized by its RNA dependent RNA polymerase (1), meaning that DNA plasmids could not be virally transcribed. One possibility would be to develop a system similar to the VIRGS plasmid, in which a DNA plasmid expresses viral RNAs harboring shRNA sequences that would be expressed in infected cells. The main problem with this approach is that the segments may simply form hairpins on their own and enter into the RNAi pathway independently of infection. An alternative approach would be to develop a plasmid that produces shRNA under control of IFNα promoter, so that shRNA is produced at the same time that interferon alpha is expressed. However, an interferon driven shRNA vector would not be effective against influenza either, because the NS1 protein has been shown to disrupt the IFN pathway during infection (83). Therefore, our current development of capped pro-siRNAs may be the only potentially viable strategy for the development of an infection dependent RNAi antiviral therapy against influenza.

If a capped pro-siRNA can be produced that can be efficiently activated in infected cells, and has similar potency to a conventional siRNA, it could provide a promising alternative to the antiviral drugs currently available. Using chemical synthesis, pro-siRNAs could be produced on an industrial scale against common seasonal influenza strains or a pandemic strain as soon as target sequences become available. One disadvantage of this approach is that the virus could develop resistance from siRNA treatments via mutation, to which a typical countermeasure might be to administer siRNAs against multiple genes or multiple sequences within a gene. Another solution to this problem would be pro-siRNAs against host genes, which may be better targets than viral genes due to their superior sequence stability. Such pro-siRNAs would also be usable against multiple influenza viruses, as the sequences of each virus would not matter so long as it could effectively cleave the pro-siRNA via PA endonuclease activity. Therefore, host-

targeting pro-siRNAs could be mass produced as a treatment against seasonal and pandemic influenza. These could be stockpiled in a similar manner to how current antivirals are held in reserve for the next pandemic. (84) Though they would not be as effective as vaccines in controlling pandemic or seasonal flu, premade stocks of viral and host targeting pro-siRNAs could provide a promising backup in the event of a mismatched seasonal vaccine, or be distributed in a pandemic while awaiting a the production of a vaccine. While our current pro-siRNAs lack sufficient potency for use in influenza antivirals, the eGFP silencing activity they do demonstrate suggests that they function as hypothesized, and that further work should be done to optimize their construction and application. With further development, pro-siRNAs could develop into a novel class of antiviral therapeutics against influenza.

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Curriculum Vitae

Residence:

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443-287-8750 (lab)

Personal Information:

Date of Birth: 10/11/1987
Place of Birth: Omaha, NE
Citizenship: US

Education:

The Johns Hopkins Bloomberg School of Public Health August 2012—May 2014 (GPA: 4.0)
Master of Science in Molecular Microbiology and Immunology
-Thesis: Utilizing Influenza 5' exonuclease activity in the development of novel RNAi therapeutics (Advisor: Dr. Andrew Pekosz)

University of Iowa College of Liberal Arts and Sciences August 2006 – May 2011 (GPA: 3.90)
Bachelor of Science in Microbiology with Honors
-Honors Thesis: The effect of single amino acids on lateral flagellar gene expression in *Vibrio parahaemolyticus* via the Scr pathway. (Advisor: Dr. Linda McCarter)

Bachelor of Arts in Music with emphasis on Trombone Performance

Honors and Awards:

- Emergent Biosolutions Fellowship Award, October 2013
  - Awarded to second year ScM student applicant studying a pathogen of global public health importance
- Graduation with Distinction (among top 10% of the University of Iowa Class of 2011)
- Allen J. Markovetz Award for Academic Excellence, May 2011
  - Awarded to the Iowa Microbiology student with the highest GPA that year
- On University of Iowa Deans List from December 2006 to May 2011.
  - Members must earn a GPA above 3.5 each semester.
- On University of Iowa Presidents List from December 2008 to December 2010.
  - Members must earn a GPA of 4.0 or above for two consecutive semesters.
- Phi Beta Kappa, November 2009
- Phi Eta Sigma, April 2007
- National Scholarship, University of Iowa

Research Experience and Skills:

Thesis Research in the laboratory of Dr. Andrew Pekosz

February 2013—May 2014

- Tissue culture (sterile technique, passaging, counting, freezing, thawing)
- Infected cells with influenza virus for experiments and for the production of virus stocks
- Titred virus stocks via TCID50 assays
- Transformed competent *E. coli* cells to produce plasmid DNA
- Prepared plasmid DNA stocks via Qiagen Mini and MaxiPreps, quantified via nanodrop.spectrophotometer and confirmed plasmid identity using restriction digests
- Transfected cells with plasmids and siRNA duplexes using lipofectamine 2000 and Nucleofection
- Used immunofluorescence for viral NS1 protein to identify infected cells via flow cytometry
- Used flow cytometry to determine the percentage of infected and transgene expressing cells
- Used flow cytometry to show that infection does not alter siRNA mediated knockdown of eGFP expression in 293T, A549 and MDCK cells.
- Designed and constructed novel siRNAs, including purification of modified ssRNAs and annealing to form duplexes
- Designed and conducted assays to screen novel siRNA constructs for eGFP silencing activity via flow cytometry
- Reported and discussed experimental results in a Thesis for Master of Science degree

Laboratory Rotation in the laboratory of Dr. J. Marie Hardwick
November 2012—February 2013

- Yeast culture (handling, freezing culture stocks, preparation of YPD and ME medias)
- Screened knockout *Saccharomyces cerevisiae* strains of interest for resistance to heat stress and overgrowth on low amino acid media.

Undergraduate Research Assistant in laboratory of Dr. Linda L. McCarter

August 2009—May 2011

- Bacterial culture (handling, freezing culture stocks, preparation of minimal media)
- Developed a semi-quantitative assay utilizing x-gal plates to compare and rank differences in gene expression in *laf::lacZ* reporter strains of *V. parahaemolyticus.*
- Examined gene expression through the use of betagalactosidase assays, crossectional and longitudinal lux assays.
- Determined that certain amino acids lead to increased expression of *laf* via the Scr system, but confirmed that the ScrA-BC synapse is not bridged by a single amino acid.
- Reported and discussed results in an Undergraduate Honors Thesis

Volunteer Technician in the laboratory of Dr. Dominic Cosgrove, Boys Town National Research Hospital, Omaha, NE

June—August 2009 and June—August 2010

- Demonstrated the difference between differentiated and non-differentiated podocytes using conventional PCR analyzed by agarose gel electrophoresis

- Demonstrated the presence of podocytes in urine of mice with Alport Syndrome via western blot, confirming decreased podocyte cell-cell adhesion as part of the Alport mode of pathology
- Trained in the isolation and purification of RNA.
- Used Reverse Transcriptase to convert purified RNA into cDNA
- Introduced to Real Time PCR

Presentation:

Sutton, Z., Cheung, L., Jesteadt, E., Meehan, D. Gratton, M. A., Delimont, D., Cosgrove, D. (2011). Derivation of conditionally immortalized clonal cell populations from strial explants derived from the immortomouse: modulation of gene expression by high glucose and hypoxia. Association for Research in Otolaryngology, Baltimore, MD.